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# *In Vitro* and Computational Evaluation of 1-O-Benzoylkarakoline on Vascular Calcium Transport

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**Abstract**

We aimed to investigate the vasorelaxant properties of 1-O-benzoylkarakoline, a semi-synthetic diterpenoid alkaloid, and to elucidate its interactions with calcium transport proteins in vascular smooth muscle. Isolated rat aortic rings were exposed to depolarization with 50 mM KCl and receptor-mediated contraction with 1 μM phenylephrine. Intracellular calcium mobilization was assessed in $Ca^{2+}$-free medium using $IP_3R$- and RyR-mediated contractions. NCX reverse mode was studied in $Na^+$-free solution, and $Na^+/K^+$-ATPase involvement was tested in ouabain-induced contraction. Endothelium dependency was examined using mechanical removal and pharmacological inhibition (L-NAME, indomethacin). Molecular docking was conducted with L-type $Ca^{2+}$ channels, RyR, SERCA, NCX1, and $Na^+/K^+$-ATPase. 1-O-benzoylkarakoline induced potent relaxation against KCl contraction (91.8 ± 3.7%, $IC_{50}$ = 2.2 μM) and phenylephrine contraction (92.1 ± 3.1%, $IC_{50}$ = 5.1 μM). It attenuated $IP_3R$-mediated contraction by 13.1 ± 2.6% and RyR-mediated contraction by 27.6 ± 4.3%. Under $Na^+$-free conditions, NCX reverse mode contraction was reduced by 22.3 ± 4.3%, while ouabain-induced contraction decreased by 28.1 ± 4.0%. Vasorelaxant effects were endothelium-independent. Docking analysis showed strong affinities with L-type $Ca^{2+}$ channels (−8.4 kcal/mol), RyR (−7.7), SERCA (−8.1), NCX1 (−8.2), and $Na^+/K^+$-ATPase (−11.8), involving hydrogen bonds, π-π stacking, π-alkyl, van der Waals, and salt bridges. 1-O-benzoylkarakoline exerts strong vasorelaxant activity by modulating calcium transport across multiple membrane and intracellular targets. Its endothelium-independent mechanism and multi-target interactions suggest therapeutic potential for vascular disorders associated with calcium overload and hypercontractility.

**Keywords:** Vasorelaxation, 1-O-benzoylkarakoline, Molecular docking, Calcium ion transport, L-type $Ca^{2+}$ channel

## Introduction

Calcium ion ($Ca^{2+}$) transport plays a pivotal role in the regulation of vascular tone, particularly in aortic smooth muscle cells, where the contraction-relaxation cycle is tightly modulated by the flux of $Ca^{2+}$ through various channels, exchangers, and intracellular stores [1]. Dysregulation of these calcium-handling systems underlies various cardiovascular disorders, including hypertension and vasospasm. Therefore, understanding how bioactive compounds modulate $Ca^{2+}$ homeostasis offers critical insight into potential therapeutic interventions. Among the key elements involved in vascular smooth muscle contraction are voltage-dependent L-type $Ca^{2+}$ channels (Cav1.2), G protein-coupled receptor (GPCR) pathways, sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA), ryanodine (RYR) and $IP_3$-sensitive receptors, sodium-calcium exchangers (NCX),

and the $Na^+/K^+$-ATPase pump [2]. These systems collectively determine intracellular calcium dynamics and vascular tone. Pharmacological agents like verapamil (a well-known L-type $Ca^{2+}$ channel blocker) and phentolamine (a non-selective α-adrenergic antagonist) have been extensively used to dissect the functional roles of these pathways [3].

In the search for novel modulators of calcium signaling, natural products, especially alkaloids, have gained attention for their structural diversity and bioactivity. Diterpenoid alkaloids, derived from plants such as Aconitum and Delphinium, exhibit a wide range of pharmacological effects, including antiarrhythmic, analgesic, and vasorelaxant properties [4]. However, their precise mechanisms of action on $Ca^{2+}$ transport in vascular smooth muscle remain underexplored. 1-O-benzoylkarakoline, a diterpenoid alkaloid, has shown potential vasomodulatory activity in preliminary screens. Therefore, its direct effects on calcium regulatory systems have not been systematically characterized.

In this study, we aimed to investigate the influence of 1-O-benzoylkarakoline on calcium ion transport in isolated rat thoracic aorta using an integrated pharmacological strategy. We employed high $K^+$-induced depolarization, GPCR activation, and intracellular $Ca^{2+}$ mobilization protocols to delineate its site-specific actions. Furthermore, we conducted comparative analyses with standard reference drugs (verapamil and phentolamine) to benchmark its pharmacological profile [5]. To complement the functional experiments, molecular docking simulations were performed to predict the binding affinity and interaction sites of 1-O-benzoylkarakoline with major calcium-handling proteins, providing a mechanistic basis for its observed effects.

This combined in vitro–in silico approach offers a comprehensive view of how 1-O-benzoylkarakoline modulates vascular smooth muscle $Ca^{2+}$ transport, potentially contributing to the development of new antihypertensive agents derived from natural products.

## Materials and methods

### Animal experiments

The experimental protocols complied with the standards and requirements for the humane treatment of animals and the provisions of the Ethical Commission of the IBB at the National University of Uzbekistan (Protocol No. 7 of 04/07/2022) on the use of laboratory animals. Preparations of isolated aortic segments were obtained using a known method. The Wistar rats (male, 220 - 250 g) were housed in a certified animal facility under standard environmental conditions: temperature $22 \pm 2$ °C, relative humidity 50% - 60%, and a 12 h light/dark cycle. Animals were provided ad libitum access to a standard pellet diet and clean drinking water. Prior to the experiments, animals were acclimatized for at least 7 days. All procedures were conducted in accordance with the principles outlined in the Guide for the Care and Use of Laboratory Animals.

### Chemical modification and structural optimization of karakoline

1-O-Benzoylkarakoline (molecular formula: $C_{29}H_{37}NO_4$; molecular weight: 481 g/mol) is a semi-synthetic derivative of the diterpenoid alkaloid karakoline, originally isolated from the plant species Aconitum karakolicum [6]. Its structure is based on the lycoctonine-type diterpenoid backbone, which was chemically modified through the selective introduction of a benzoyl group at the C(1)-hydroxyl position. This structural modification was carried out to enhance the pharmacological activity of the parent compound **Figure 1**. The synthesis of 1-O-benzoylkarakoline was guided by preliminary pharmacological screening, which demonstrated that substitution at various positions of the lycoctonine skeleton significantly influences the biological activity profile. In particular, the incorporation of a benzoyl group at position C(1) of karakoline markedly enhanced its vasorelaxant effect in comparison to the unmodified parent compound. These results show that making small chemical changes, like adding acyl groups to certain hydroxyl positions, could be a useful way to improve the biological activity of natural diterpenoid alkaloids. All reagents and solvents used in the synthesis and purification of 1-O-benzoylkarakoline were of analytical grade. Purity of the final compound was confirmed by chromatographic and spectroscopic methods (e.g., TLC, NMR, MS) prior to use in pharmacological and docking experiments.

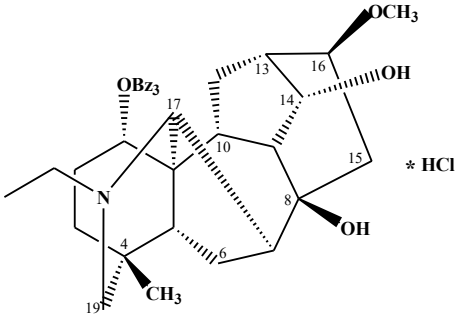


**Figure 1** Chemical structure of 1-O-benzoylkarakoline (1-O-BK), a semi-synthetic diterpenoid alkaloid derived from karakoline.

**Chemicals**

The chemical agents phenylephrine, phentolamine, ouabain, choline chloride and verapamil were procured from Sigma-Aldrich Chemie, a division of Sigma-Aldrich, based in St. Louis, MO, USA.

**Tissue preparation**

Surgery was performed under sodium pentobarbital anesthesia to reduce distress. Experiments were performed on aortic tissue from healthy white male rats (200 - 250 g). Animals were killed by cervical dislocation. The thoracic cavity was opened, and the aorta was removed, cleaned of adhering fat and connective tissue, and cut into 3 - 4 mm segments. The aortic rings were put in a 5 mL organ bath filled with Krebs-Henseleit physiological solution with (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-buffered to pH 7.4. For certain experiments, a Krebs solution free of $Ca^{2+}$ and with 1 mM EGTA was used. The bath solution was continuously gassed with carbogen (95% $O_2$ and 5% $CO_2$) and maintained at 37 °C using a DAIHAN WATER BATH ultrathermostat [7].

**Aortic-ring contraction studies**

The aortic rings were suspended in a Radnoti isometric transducer system (USA) using platinum wire hooks and equilibrated for 60 min under normal conditions. The preparations were all stretched with an initial load of 1 g (10 mN). Contractile responses were transferred from the transducer to a signal amplifier and were digitally recorded by a Go-link automatic converter interfaced with a computer. Data were analyzed with Origin Pro v.9 SR1 (EULA, Northampton, MA, USA). The isometric contraction force (mN) of the rat aortic tissue in vitro was calculated as a percentage (%) for statistical analysis. The preparations of vascular smooth muscle were studied on an apparatus. The organ bath (5 mL) was connected via a special reservoir circulating the Krebs-Henseleit physiological solution **Figure 2**. The physiological temperature was constantly maintained with the help of a thermostat, and the solution was continuously gassed with a mixture of 95% $O_2$ and 5% $CO_2$. The contractile activity of the aortic vascular segment, mounted in the experimental chamber, was recorded with the help of an ISOMETRIC TRANSDUCER (Grass Instrument, USA) and displayed on the GoLink signal amplifier and support system [8].

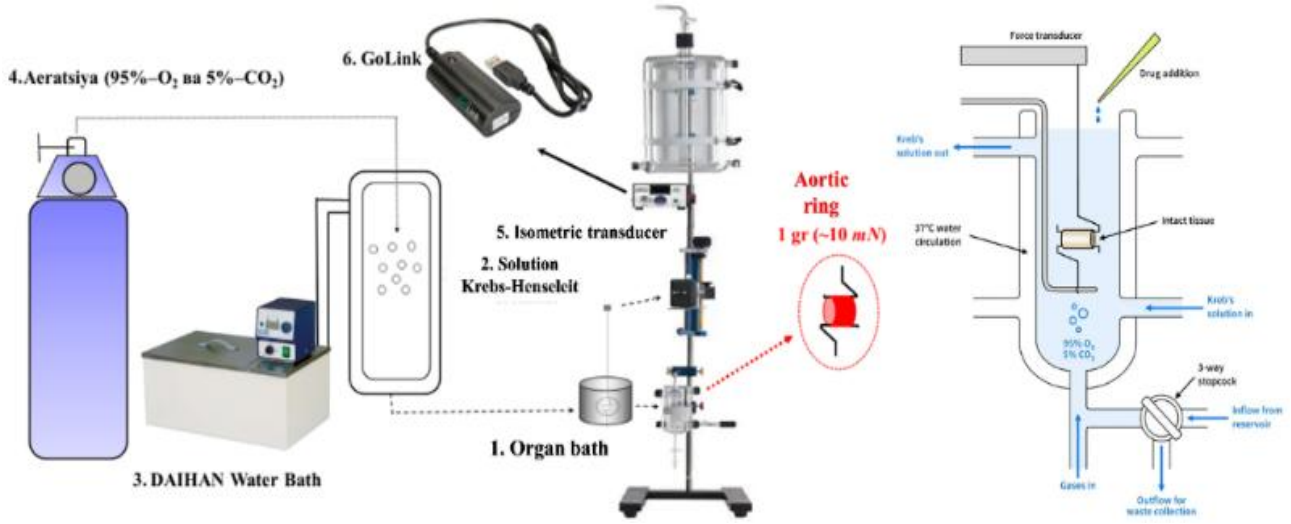


**Figure 2** Schematic representation of the organ bath system used for isometric tension recording of isolated rat aortic rings. The setup includes (1) organ bath chamber maintained at 37 °C by (3) a circulating water bath (DAIHAN), (2) filled with Krebs-Henseleit solution, (4) continuously aerated with a 95% $O_2$ and 5% $CO_2$ gas mixture, and (5) connected to an isometric force transducer to detect contractile responses. The data is acquired through (6) a GoLink interface. The aortic ring (~1 g tension ≈ 10 mN) is suspended between 2 hooks within the chamber (right), allowing for precise drug addition and real-time measurement of smooth muscle responses under controlled physiological conditions.

**Statistical analysis**

All statistical analyses and graphical data presentations were carried out using Origin Pro 9 software (Microsoft, USA). Vascular contractile responses were quantified as a percentage relative to the maximal contraction elicited by phenylephrine (10 mM) or potassium chloride (KCl, 50 mM). Data are presented as mean values derived from 5 to 8 independent experiments (n = 3 - 6). Paired t-tests were employed for within-group comparisons, while unpaired t-tests were used to evaluate differences between separate experimental groups. Statistical significance was set at $p < 0.05$.

**Molecular docking: Software and databases**

All software tools used in this study are freely accessible for academic purposes. Structural information on biomacromolecules related to calcium signaling and regulation was retrieved from the Protein Data Bank (PDB), a well-established database of 3-dimensional biomolecular structures [9,10]. The target proteins selected for analysis included the L-type 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 ryanodine receptor RyR2 (PDB ID: 5c33), and the sarcoplasmic/endoplasmic reticulum calcium ATPase SERCA (PDB ID: 6rb2).

In addition, the PubChem database was utilized to retrieve structures of natural flavonoids and standard reference compounds. PubChem integrates a wealth of pharmacological, chemical, biochemical, and molecular data, including ligand structures, sequences, pathways, and associated protein targets. Each compound entry in PubChem includes extensive metadata (over 80 descriptors) covering chemical, structural, and bioactivity information, as summarized in **Table 1**. Three-dimensional visualization and structural inspection of the downloaded PDB files were performed using PyMOL (version 1.2), a Python-based molecular graphics software. PyMOL was also used for rendering and interpreting docking outcomes. For molecular docking simulations, AutoDock 4.2 - developed by The Scripps Research Institute - was employed. Ligand and receptor preparation, along with the setup of docking parameters, was carried out using AutoDock Tools (ADT), a graphical interface that provides convenient control over the docking workflow. AutoDock enables the prediction of binding modes and interaction energies of small molecules with target macromolecules, providing insight into potential ligand–protein interactions at the atomic level.

## Results and discussion

### Evaluation of the effect of 1-O-benzoylkarakoline on KCl-induced contraction via L-type $Ca^{2+}$ channels

It is well established that depolarization of vascular smooth muscle cells using potassium chloride (KCl, 50 mM) leads to the opening of voltage-dependent L-type calcium channels, resulting in calcium influx and sustained contraction. To investigate whether 1-O-benzoylkarakoline (1-O-BK) exerts its vasorelaxant effect through modulation of these channels, we examined its influence on KCl-precontracted rat aortic rings [11,12].

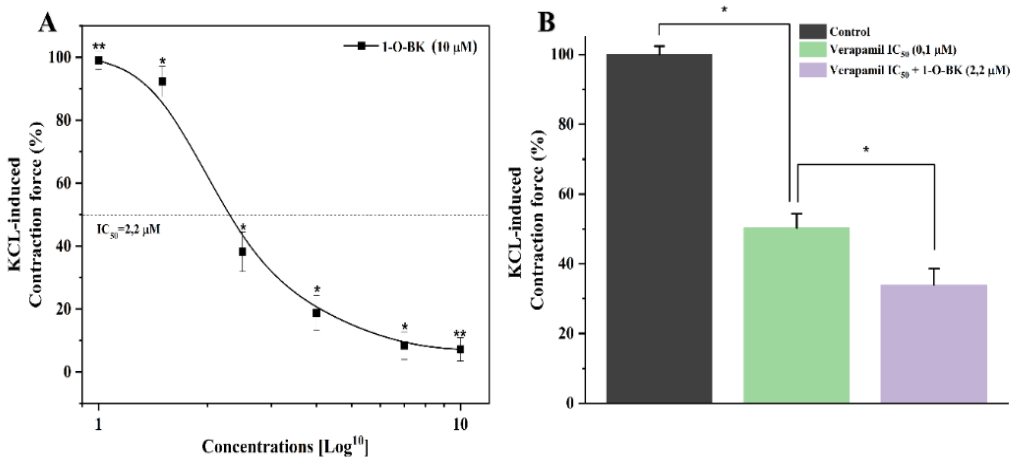


**Figure 3** Inhibitory effect of 1-O-benzoylkarakoline (1-O-BK) on KCl-induced contraction of rat aortic rings via L-type $Ca^{2+}$ channels. (A) Concentration-response curve of 1-O-BK (1 - 30 µM) on KCl (50 mM)-induced contractions. 1-O-BK produced a concentration-dependent relaxation, with an $IC_{50}$ value of 2.2 µM. (B) Comparative analysis showing the contractile force in control, verapamil (0.1 µM), and combined treatment (verapamil + 1-O-BK, both at $IC_{50}$). Co-administration significantly enhanced the inhibitory effect, suggesting potential synergy in targeting L-type $Ca^{2+}$ channels. Data are expressed as mean ± SEM (n = 5 - 6). *$p < 0.05$, **$p < 0.01$ vs. control.

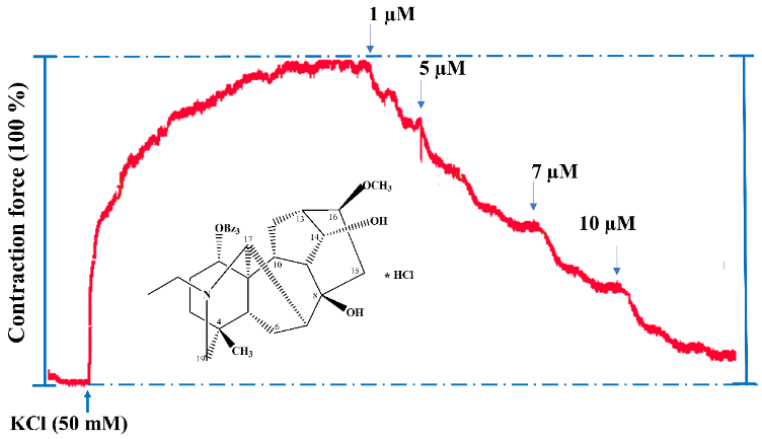


**Figure 4** Original isometric tension recording demonstrating the vasorelaxant effect of 1-O-benzoylkarakoline on rat aortic rings pre-contracted with 50 mM KCl. Stepwise additions of the compound (1, 5, 7 and 10 µM) led to a dose-dependent decrease in contraction force (%). The chemical structure of 1-O-benzoylkarakoline is displayed in the inset.

Cumulative administration of 1-O-BK at concentrations ranging from 10 to 150 µM produced a marked, concentration-dependent relaxation of KCl-induced contractions [Figure 4]. The extent of relaxation ranged from 38.3 ± 4.1% at 10 µM to 91.82 ± 3.7% at 150 µM, indicating a potent inhibitory effect on voltage-gated calcium entry mechanisms [Figure 3A]. To further assess whether this relaxant effect is mediated

specifically through L-type $Ca^{2+}$ channels, comparative pharmacological analysis was performed using verapamil, a classical L-type calcium channel blocker. Preincubation with verapamil at a submaximal concentration (0.1 μM) alone reduced the KCl-induced contraction by 50.0 ± 2.2%. Under the same conditions, co-application of 1-O-benzoylkarakoline led to an additional 19.5 ± 3.2% reduction in contractile force, while karakoline (the parent compound) induced only a minor additional relaxation of 3.5 ± 3.2%, which was not statistically significant (**Figure 3(B)**). These data suggest a potential synergistic or additive interaction between 1-O-BK and verapamil, highlighting the likelihood that 1-O-BK shares a similar mechanism of action, primarily targeting L-type calcium channels. Importantly, the parent compound karakoline did not exhibit significant activity under verapamil incubation, suggesting that the benzoylation at position C(1) is a critical structural modification responsible for enhanced channel inhibition. Taken together, these findings strongly indicate that the vasorelaxant activity of 1-O-benzoylkarakoline is largely mediated via inhibition of $Ca^{2+}$ influx through voltage-dependent L-type $Ca^{2+}$ channels, leading to decreased intracellular calcium concentrations and subsequent relaxation of vascular smooth muscle. These mechanistic insights position 1-O-BK as a promising candidate for further exploration in vascular pharmacotherapy, particularly in conditions characterized by enhanced calcium channel activity.

**Effect of 1-O-benzoylkarakoline on receptor-operated $Ca^{2+}$ channels activated via GPCR signaling**

To determine whether the relaxant effect of 1-O-benzoylkarakoline (1-O-BK) involves inhibition of receptor-operated $Ca^{2+}$ entry mechanisms, we investigated its influence on smooth muscle contraction induced by phenylephrine (PE), an $\alpha_1$-adrenergic receptor agonist that activates G protein-coupled receptor (GPCR)-mediated calcium signaling (**Figure 6**). In isolated rat aortic rings precontracted with phenylephrine (1 μM), cumulative administration of 1-O-BK in the concentration range of 1 - 30 μM produced a robust and dose-dependent relaxation [13,14]. At 30 μM, 1-O-BK significantly suppressed PE-induced contraction by 92.1 ± 3.1%, with an estimated half-maximal inhibitory concentration ($IC_{50}$) of 5.1 μM. These results show that 1-O-BK strongly reduces GPCR-driven vasoconstriction, probably by blocking calcium entry through receptor-operated pathways **Figure 5(A)**.

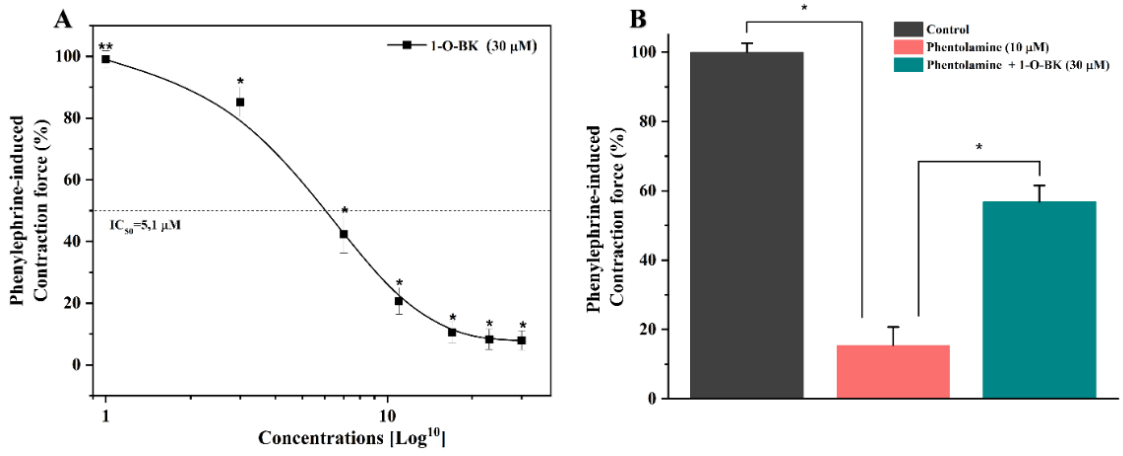


**Figure 5** Inhibitory effect of 1-O-benzoylkarakoline (1-O-BK) on phenylephrine-induced contraction via receptor-operated $Ca^{2+}$ entry in rat aortic rings. (A) Concentration-response curve showing the vasorelaxant effect of 1-O-BK (1 - 30 μM) on contractions induced by phenylephrine (1 μM). The compound elicited a dose-dependent relaxation with an $IC_{50}$ value of 5.1 μM. (B) Comparative analysis of phenylephrine-induced contractions under control conditions, with phentolamine (10 μM), and with combined phentolamine + 1-O-BK (30 μM). Co-administration significantly reduced contractile force, indicating that 1-O-BK may modulate $\alpha_1$-adrenergic receptor-mediated $Ca^{2+}$ influx. Data are expressed as mean ± SEM (n = 5 - 6). *$p < 0.05$ vs. control.

To further clarify the specificity of this mechanism, we examined the effect of phentolamine (10 µM), a well-known non-selective α-adrenergic receptor antagonist, on the relaxant response of 1-O-BK. In the absence of phentolamine, 1-O-BK (30 µM) reduced PE-induced contraction by over 92%, as previously observed. However, in the presence of phentolamine, the relaxant effect of 1-O-BK was markedly attenuated, resulting in only 41.4 ± 4.7% inhibition compared to control (**Figure 5(B)**). This substantial reduction in efficacy in the presence of an α-receptor blocker confirms that the vasorelaxant activity of 1-O-BK is significantly mediated via $\alpha_1$-adrenergic receptor-dependent signaling pathways, which are closely associated with receptor-operated $Ca^{2+}$ channels. The comparison highlights that 1-O-BK likely disrupts calcium influx secondary to GPCR activation, providing mechanistic insight into its dual modulatory action on both voltage-operated and receptor-operated calcium entry systems. These findings suggest that 1-O-benzoylkarakoline could be a useful vasorelaxant drug, since it can act on different calcium entry pathways that control vascular smooth muscle contraction.

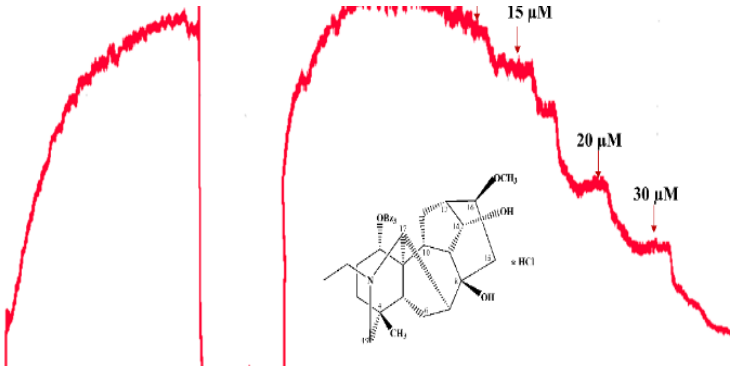


**Figure 6** Original isometric tension tracing showing the vasorelaxant effect of 1-O-benzoylkarakoline on phenylephrine (Phe, 1 µM)-precontracted isolated rat aortic rings. Cumulative additions of 1-O-benzoylkarakoline (5, 15, 20 and 30 µM) induced a stepwise decrease in contraction force, indicating dose-dependent relaxation.

**Modulatory effect of 1-O-benzoylkarakoline on $IP_3R$-mediated intracellular $Ca^{2+}$ release under calcium-free conditions**

To further investigate the mechanism underlying the vasorelaxant activity of 1-O-benzoylkarakoline (1-O-BK), we assessed its effect on receptor-operated intracellular $Ca^{2+}$ release via inositol-1,4,5-trisphosphate receptors ($IP_3R$) in a calcium-free extracellular environment ($[Ca^{2+}]_{out}$ = 0 mM). According to the literature, under such conditions, smooth muscle contraction can still occur through the mobilization of $Ca^{2+}$ from intracellular stores, particularly from the sarcoplasmic reticulum via $IP_3R$ activation following stimulation with phenylephrine (PE). In our experiment, phenylephrine (1 µM) was used to induce receptor-mediated contraction in calcium-free Krebs solution, and the effect of 1-O-BK was evaluated under these conditions. Treatment with 1-O-BK (30 µM) resulted in a modest but statistically significant reduction in PE-induced contraction by 13.1 ± 2.6% compared to the control [15,16]. This suggests that 1-O-BK partially interferes with intracellular $Ca^{2+}$ release mediated by $IP_3R$ activation, indicating a regulatory effect on internal $Ca^{2+}$ mobilization mechanisms in vascular smooth muscle. To further confirm the involvement of sarcoplasmic $Ca^{2+}$ release, additional experiments were performed using caffeine (10 mM), a known activator of ryanodine receptors (RyR), which induces transient contraction by triggering $Ca^{2+}$ efflux from the sarcoplasmic reticulum. In standard extracellular calcium conditions ($[Ca^{2+}]_{out}$ = 2.5 mM), caffeine induced a contractile response of 65.4 ± 4.1% relative to the KCl control. When aortic tissues were pretreated with varying concentrations of 1-O-BK (1 - 30 µM), a dose-dependent attenuation of caffeine-induced contraction was observed, with maximal inhibition reaching 27.6 ± 4.3% at 30 µM. These results together suggest that 1-O-benzoylkarakoline can attenuate intracellular calcium release from the sarcoplasmic reticulum, likely by modulating both $IP_3R$- and RyR-

mediated pathways. Although the inhibitory effect under calcium-free conditions was moderate compared to extracellular influx blockade, these findings provide further evidence that 1-O-BK exerts multifaceted control over calcium signaling, not only by blocking plasma membrane L-type channels but also by interfering with internal calcium release systems that govern vascular tone.

**Effect of 1-O-benzoylkarakoline on caffeine-induced $Ca^{2+}$ release via ryanodine receptors (RyR) in the presence and absence of extracellular $Ca^{2+}$**

To further verify the involvement of intracellular calcium stores in the vasorelaxant effect of 1-O-benzoylkarakoline (1-O-BK), we examined its influence on $Ca^{2+}$ release mediated through ryanodine receptors (RyR), using caffeine as a pharmacological activator. Caffeine (10 mM) is well-documented to induce transient contraction in vascular smooth muscle by triggering calcium release from the sarcoplasmic reticulum via RyR activation [17,18]. In our experiments, application of caffeine in normal Krebs solution ($[Ca^{2+}]_{out}$ = 2.5 mM) produced robust aortic contractions, reaching 65.4 ± 4.1% relative to the standard Phenylephrine-induced contraction [Figure 7A]. Preincubation with increasing concentrations of 1-O-BK (1–30 µM) resulted in a dose-dependent attenuation of this caffeine-induced response, with a maximal reduction of 27.6 ± 4.3% and an estimated $IC_{50}$ value of 16.6 µM, indicating that 1-O-BK effectively suppresses calcium release via RyR under physiological conditions. To dissect the role of extracellular calcium influx in this response, parallel experiments were conducted under calcium-free conditions ($[Ca^{2+}]_{out}$ = 0 mM). Under these conditions, caffeine still induced a measurable contraction (33.3 ± 3.4%), reflecting $Ca^{2+}$ mobilization solely from intracellular stores. Notably, in the absence of extracellular calcium, pretreatment with 1-O-BK (30 µM) significantly reduced caffeine-induced contraction to 18.6 ± 4.3%, with an $IC_{50}$ value of 18.2 µM [Figure 7B]. These findings indicate that 1-O-benzoylkarakoline attenuates calcium release from the sarcoplasmic reticulum via RyR activation, both in the presence and absence of extracellular calcium. This effect may be due to changes in the cell's calcium stores or blocking signals that control RyR-driven calcium release. The observed decrease in contraction amplitude under both conditions supports the hypothesis that 1-O-BK contributes to the regulation of intracellular calcium homeostasis, possibly by inhibiting $Ca^{2+}$ reuptake into the sarcoplasmic reticulum or enhancing $Ca^{2+}$ extrusion from the cytosol.

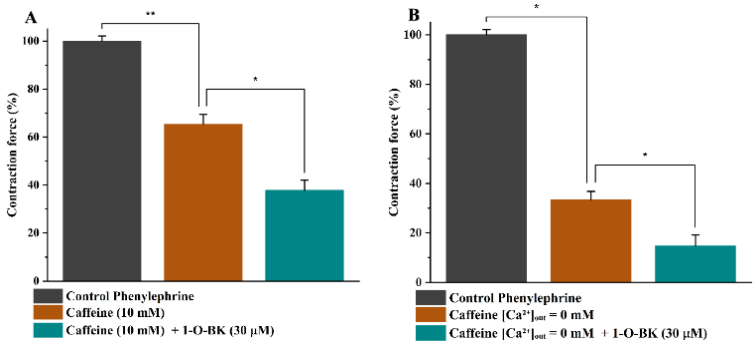


**Figure 7** Inhibitory effect of 1-O-benzoylkarakoline (1-O-BK) on caffeine-induced contraction of rat aortic rings via RyR-mediated intracellular $Ca^{2+}$ release. (A) Caffeine (10 mM) induced significant contraction compared to phenylephrine control. Preincubation with 1-O-BK (30 µM) significantly attenuated caffeine-induced contraction, suggesting inhibition of calcium release via ryanodine receptors (RyRs). (B) Under calcium-free conditions ($[Ca^{2+}]_{out}$ = 0 mM), caffeine-induced contraction was reduced compared to the control. Co-application of 1-O-BK (30 µM) further suppressed the contractile response, indicating that 1-O-BK interferes with intracellular $Ca^{2+}$ mobilization even in the absence of extracellular calcium. Data are presented as mean ± SEM (n = 5 - 6). *$p < 0.05$, **$p < 0.01$ vs. control.

Taken together, these results demonstrate that the vasorelaxant effect of 1-O-BK extends beyond membrane-bound $Ca^{2+}$ influx pathways and includes significant modulation of intracellular $Ca^{2+}$ dynamics, specifically through the RyR-mediated release mechanism.

**Effect of 1-O-benzoylkarakoline on $Na^+/Ca^{2+}$ exchanger and $Na^+/K^+$-ATPase activity in vascular smooth muscle**

To assess the possible involvement of the $Na^+/Ca^{2+}$ exchanger (NCX) and the $Na^+/K^+$-ATPase pump in the vasorelaxant mechanism of 1-O-benzoylkarakoline (1-O-BK), we conducted a series of experiments using modified Krebs solutions and selective pharmacological inhibitors.

***Modulation of NCX activity in sodium-free conditions***

Under physiological conditions, the NCX plays a key role in maintaining intracellular calcium balance by exchanging intracellular $Ca^{2+}$ for extracellular $Na^+$. To evaluate whether 1-O-BK influences this mechanism, we induced aortic contractions using phenylephrine (1 μM) in a $Na^+$-free Krebs solution, where NaCl was replaced with equimolar choline chloride, thereby reversing the exchanger's function and promoting $Ca^{2+}$ entry into the cytosol [19].

In this setup, the phenylephrine-induced contraction in the $Na^+$-free condition reached 53.4 ± 3.6% of the control value. Subsequent application of 1-O-BK at concentrations ranging from 5 to 30 μM caused a dose-dependent inhibition of this contraction, with a maximum reduction of 22.3 ± 4.3% and an estimated $IC_{50}$ of 11.1 μM. These findings suggest that 1-O-BK effectively modulates NCX activity by limiting reverse-mode $Ca^{2+}$ influx (**Figure 8(A)**).

To test this, we used KB-R7943, a drug that blocks NCX. On its own, KB-R7943 reduced muscle contraction by about 20%. When combined with 1-O-BK, the reduction was much stronger (about 40%), showing that blocking NCX plays an important role in how 1-O-BK relaxes the muscle (**Figure 8(B)**).

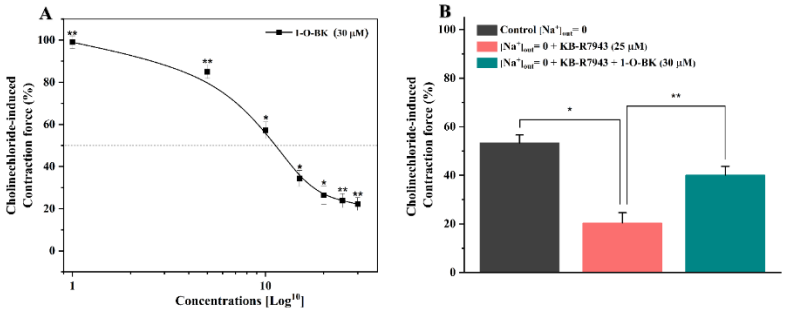


**Figure 8** Inhibitory effect of 1-O-benzoylkarakoline (1-O-BK) on choline chloride-induced contraction in $Na^+$-free conditions: Role of reverse-mode $Na^+/Ca^{2+}$ exchanger (NCX). (A) Concentration-response curve showing the dose-dependent relaxation effect of 1-O-BK (1 - 30 μM) on phenylephrine-induced contraction in $Na^+$-free Krebs solution (NaCl replaced with choline chloride), where NCX operates in reverse mode, promoting $Ca^{2+}$ entry. 1-O-BK markedly attenuated contraction with an $IC_{50}$ value of 11.1 μM. (B) Bar graph comparing contraction force under $Na^+$-free conditions with and without NCX inhibition by KB-R7943 (25 μM) and their combination with 1-O-BK (30 μM). Co-administration significantly reduced contraction amplitude, confirming that 1-O-BK modulates $Ca^{2+}$ influx through reverse-mode NCX. Data are shown as mean ± SEM (n = 5 - 6). *$p < 0.05$, **$p < 0.01$ vs. control.

***Evaluation of $Na^+/K^+$-ATPase-mediated regulation via ouabain challenge***

To investigate whether $Na^+/K^+$-ATPase participates in the regulation of NCX and intracellular $Ca^{2+}$ dynamics under the influence of 1-O-BK, ouabain - a known cardiac glycoside and $Na^+/K^+$-ATPase inhibitor - was used. Ouabain raises the level of $Na^+$ inside the cell, which causes NCX to work in reverse. As a result, more $Ca^{2+}$ builds up, leading to stronger contractions [20,21].

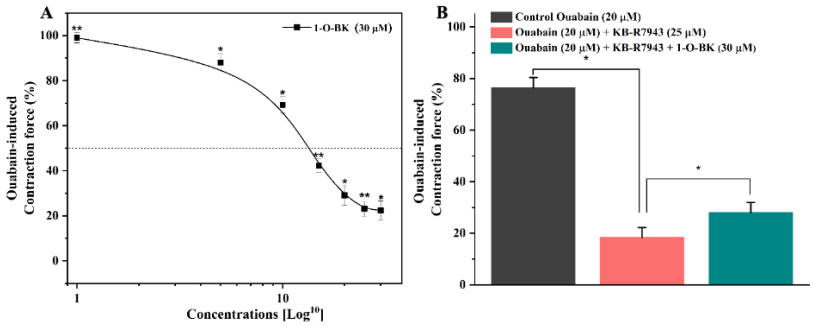


**Figure 9** Inhibitory effect of 1-O-benzoylkarakoline (1-O-BK) on ouabain-induced contraction of rat aortic rings: Involvement of $Na^+/K^+$-ATPase and $Na^+/Ca^{2+}$ exchanger (NCX). (A) Concentration-response curve showing the dose-dependent vasorelaxant effect of 1-O-BK (1 - 30 μM) on contractions induced by ouabain (20 μM), with an estimated $IC_{50}$ value indicating significant inhibition of $Na^+/K^+$-ATPase-mediated calcium accumulation. (B) Comparative analysis of contraction force under control conditions, ouabain + KB-R7943 (25 μM; NCX inhibitor), and ouabain + KB-R7943 + 1-O-BK (30 μM). Co-administration significantly reduced the contractile force, suggesting that 1-O-BK acts synergistically with NCX inhibition to attenuate calcium overload induced by ouabain. Data are presented as mean ± SEM (n = 5 - 6). *$p < 0.05$, **$p < 0.01$ vs. control.

In this model, 1-O-BK (30 μM) significantly attenuated ouabain-induced aortic contractions, producing a 22.3 ± 4.1% reduction compared to control, with an estimated $IC_{50}$ of 10.5 μM. This effect was notably diminished when KB-R7943 was added to the system: The NCX inhibitor alone reduced ouabain-induced contractions by 18.4 ± 4.1%, and 1-O-BK under these conditions showed a reduced relaxant effect, with contraction amplitude only decreasing by 28.1 ± 4.0% (**Figure 9(A)**).

These complementary results suggest that 1-O-benzoylkarakoline exerts its relaxant effect in part through inhibition of $Ca^{2+}$ influx mediated by the reverse-mode $Na^+/Ca^{2+}$ exchanger, and that this process is modulated via the $Na^+/K^+$-ATPase-NCX axis. The diminished contractility observed under combined treatment conditions (ouabain + KB-R7943 + 1-O-BK) further supports the notion that 1-O-BK interferes with the complex regulation of intracellular $Ca^{2+}$ via both membrane transporters, offering additional mechanistic insight into its vasorelaxant properties **(Figure 9(B))**.

**Evaluation of endothelium-dependent mechanisms in the vasorelaxant effect of 1-O-benzoylkarakoline**

To assess whether the vasorelaxant effect of 1-O-benzoylkarakoline (1-O-BK) involves endothelium-dependent pathways, we investigated its activity on isolated rat aortic rings under both intact and endothelium-denuded conditions [22,23]. Vascular rings were precontracted with phenylephrine (1 μM) or KCl (50 mM) to induce sustained contraction, and then treated with cumulative concentrations of 1-O-BK. In endothelium-intact preparations, 1-O-BK produced a concentration-dependent relaxation, ranging from 15.3 ± 3.6% to a maximal 92.1 ± 3.1% at 30 μM. The half-maximal inhibitory concentration ($IC_{50}$) of 1-O-BK was determined to be 2.2 μM under KCl-induced contraction and 5.1 μM under phenylephrine-induced contraction. To determine the role of the vascular endothelium in mediating this effect, the endothelial layer was mechanically removed. In endothelium-denuded rings, 1-O-BK at 30 μM still induced a pronounced relaxation of 90.8 ± 2.9%, which was only slightly lower than the response observed in endothelium-intact tissues (92.1 ± 3.5%). This marginal difference suggests that the majority of the relaxant activity is independent of endothelial function (**Figure 10(A)**).

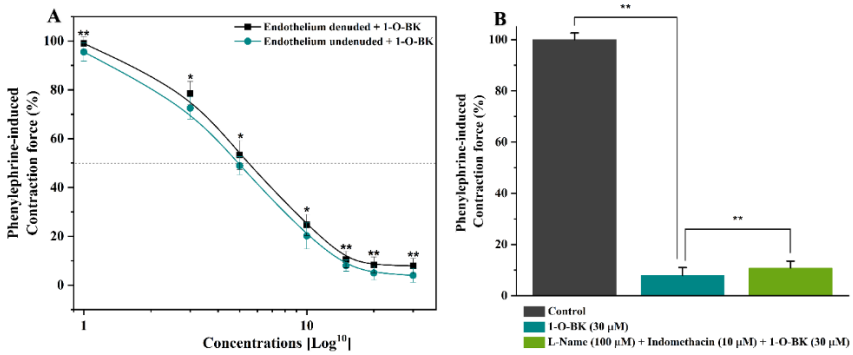


**Figure 10** Endothelium-independent vasorelaxant effect of 1-O-benzoylkarakoline (1-O-BK) on phenylephrine-induced contraction in isolated rat aortic rings. (A) Concentration-response curves comparing the relaxant effect of 1-O-BK (1 - 30 μM) on phenylephrine (1 μM)-induced contractions in endothelium-intact and endothelium-denuded aortic rings. The overlap of curves indicates that endothelium removal had minimal impact on the vasorelaxant efficacy of 1-O-BK. (B) Evaluation of the effect of nitric oxide synthase (eNOS) inhibitor L-NAME (100 μM) and cyclooxygenase inhibitor indomethacin (10 μM) on the relaxant activity of 1-O-BK (30 μM). No significant difference was observed between 1-O-BK alone and in combination with both inhibitors, supporting the conclusion that its relaxant effect is largely endothelium-independent. Data are presented as mean ± SEM (n = 5 - 6). $^{**}p < 0.01$ vs. control.

Further confirmation was obtained through pharmacological inhibition of key endothelial signaling pathways. Preincubation with L-NAME (100 μM), a nitric oxide synthase (eNOS) inhibitor, and indomethacin (10 μM), a cyclooxygenase (COX) inhibitor, did not significantly alter the relaxant effect of 1-O-BK. Under these conditions, 1-O-BK reduced phenylephrine-induced contraction by 90.1 ± 4.3%, compared to 92.1 ± 3.7% in the absence of inhibitors [Figure 10B].

Collectively, these findings clearly demonstrate that the vasorelaxant activity of 1-O-benzoylkarakoline is predominantly endothelium-independent and is likely mediated by direct action on vascular smooth muscle cells, particularly through modulation of calcium ion transport systems.

**Structure-based interactions between 1-o-benzoylkarakoline and ion transport systems**

***Molecular docking of 1-O-benzoylkarakoline with L-type $Ca^{2+}$ channel (PDB ID: 6JP5)***

To explore the binding characteristics of 1-O-benzoylkarakoline with voltage-dependent L-type calcium channels, molecular docking was performed using the crystal structure of the human CaV1.2 channel (PDB ID: 6JP5) retrieved from the Protein Data Bank. This channel serves as a central regulator of calcium influx in cardiac and vascular smooth muscle cells and represents a key pharmacological target in cardiovascular therapy [24,25].

The docking simulations were carried out using AutoDock Vina, with a predefined grid box centered at coordinates X = 172.744 Å, Y = 226.637 Å, Z = 199.228 Å and dimensions of 90×90×90 Å, encompassing the channel's active site. The binding pocket was identified using Discovery Studio based on known ligand-accessible cavities and conserved pharmacophoric regions. Ten binding poses were generated for 1-O-benzoylkarakoline, and the most energetically favorable conformation (lowest ΔG) was selected for further analysis.

The docking results revealed a binding affinity of −8.4 kcal/mol, indicating a strong and stable interaction between 1-O-benzoylkarakoline and the $Ca^{2+}$ channel. The ligand was found to engage in van der Waals and covalent interactions primarily with ASN A:257, suggesting that this residue plays a critical role in stabilizing the ligand at the channel's active site. The ligand conformation aligned well within the central pore region, supporting its potential role as a modulator of calcium conductance (**Figure 11**).

These findings suggest that 1-O-benzoylkarakoline exhibits high binding specificity and favorable energetics for targeting the L-type $Ca^{2+}$ channel, consistent with its observed in vitro vasorelaxant activity.

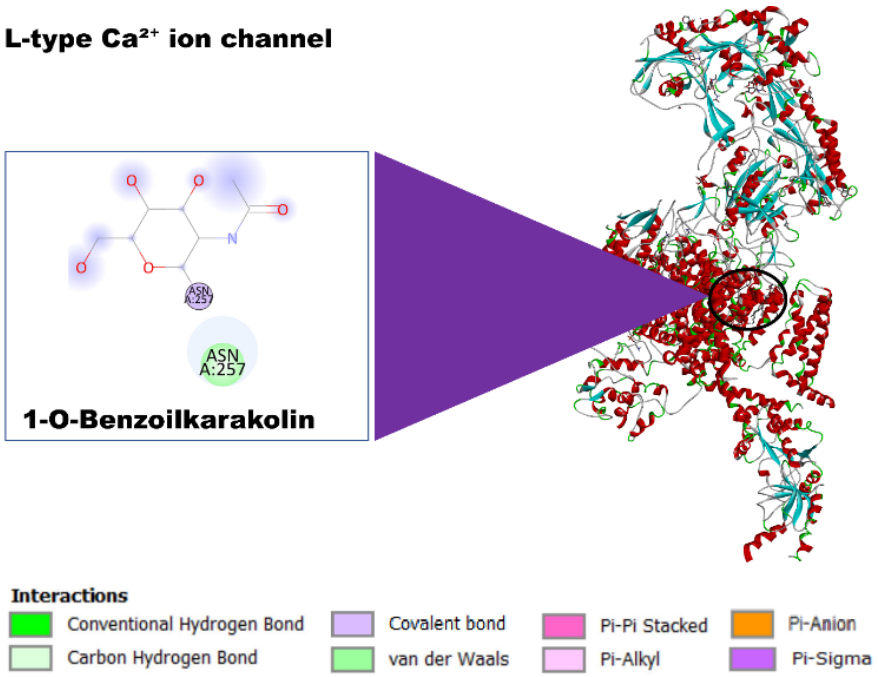


**Figure 11** Molecular docking interaction of 1-O-benzoylkarakoline with the L-type $Ca^{2+}$ ion channel (PDB ID: 6JP5). The right panel shows the ribbon structure of the channel, highlighting the ligand-binding site. The zoomed-in view on the left depicts the interaction between 1-O-benzoylkarakoline and the ASN A:257 amino acid residue via a conventional hydrogen bond. Interaction types are color-coded as indicated in the legend below.

***Molecular docking analysis of 1-O-benzoylkarakoline with ryanodine receptor (RyR)***

In this part of the study, the molecular docking interaction between 1-O-benzoylkarakoline and the ryanodine receptor (RyR) was analyzed. RyR is an intracellular calcium channel located on the sarcoplasmic reticulum membrane and plays a critical role in regulating cytosolic calcium levels. Its pharmacological modulation has therapeutic significance in controlling cardiac rhythm, smooth muscle contractility, and calcium-dependent signaling.

The crystal structure of the human RyR protein (PDB ID: 5C33) was used for docking, and the ligand structure of 1-O-benzoylkarakoline was geometrically optimized in Avogadro before being introduced into AutoDock Vina. The docking analysis revealed a binding affinity of −7.7 kcal/mol, indicating a thermodynamically favorable and stable interaction (**Figure 12**).

Importantly, 1-O-benzoylkarakoline formed conventional hydrogen bonds with several key amino acid residues in the active site of RyR, including LEU A:92, MET A:107, THR A:77 and ASN A:75. These polar interactions contribute significantly to the stability and specificity of the ligand-receptor complex. The binding conformation of the ligand was well aligned within the receptor pocket, supporting its potential role in modulating RyR-mediated calcium release. These interactions, together with the strong binding energy, suggest that 1-O-benzoylkarakoline is a potential modulator of RyR function, supporting its vasorelaxant effect by influencing both extracellular and intracellular calcium regulation [26,27].

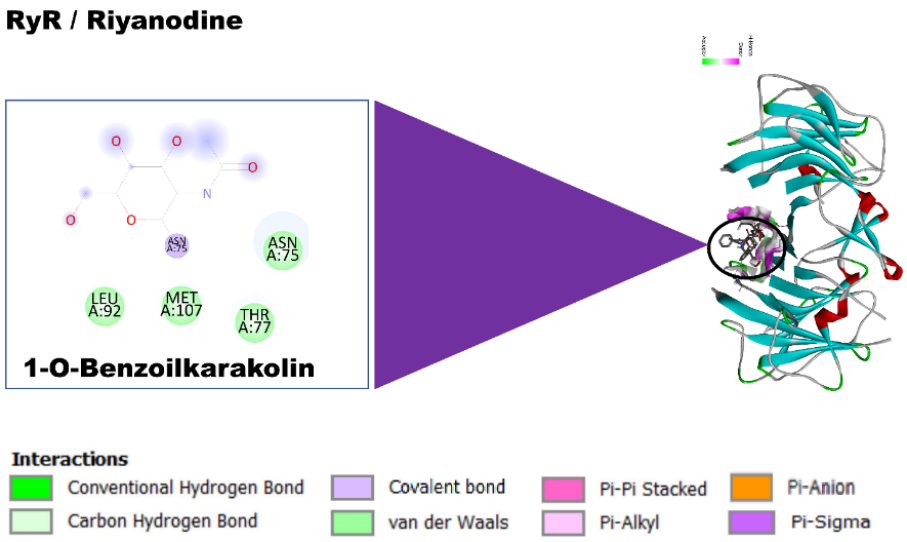


**Figure 12** Molecular docking interaction of 1-O-benzoylkarakoline with the RyR (ryanodine) receptor (PDB ID: 5C33). The ribbon diagram on the right shows the RyR protein structure with the ligand-binding pocket highlighted. The zoomed-in view on the left illustrates the specific interactions of 1-O-benzoylkarakoline with amino acid residues LEU A:92, MET A:107, THR A:77, and ASN A:75 via conventional hydrogen bonds. Interaction types are indicated by color codes as shown in the legend.

***Molecular docking analysis of 1-O-benzoylkarakoline with sarcoplasmic reticulum $Ca^{2+}$-ATPase (PDB ID: 6JJU)***

In the next phase of the docking investigation, the interaction between 1-O-benzoylkarakoline and the $Ca^{2+}$-ATPase ion channel (PDB ID: 6JJU) was evaluated. This membrane-bound enzyme, located in the sarcoplasmic reticulum of muscle cells, plays a critical role in actively transporting cytosolic $Ca^{2+}$ back into the sarcoplasmic stores, thus regulating intracellular calcium homeostasis and muscle relaxation [28,29].

The 3-dimensional structure of the $Ca^{2+}$-ATPase was retrieved from the Protein Data Bank, and the ligand 1-O-benzoylkarakoline was geometrically optimized using Avogadro software to ensure a stable low-energy conformation before docking. Molecular docking was performed using AutoDock Vina, which calculated a binding affinity of −8.1 kcal/mol, suggesting a strong and energetically favorable interaction [30,31].

Detailed interaction analysis revealed that 1-O-benzoylkarakoline formed conventional hydrogen bonds with key residues THR A:353, THR A:624, GLY A:625, and ARG A:489, which contributed to the stability of the complex. Additionally, a variety of van der Waals and carbon-hydrogen interactions were observed with ASP A:351, ASN A:705, ASP A:626, LYS A:492, GLU A:442, ALA A:516, GLY A:515, LEU A:561, SER A:493, and LYS A:514.

Furthermore, a π-π stacking interaction was identified between the aromatic ring of the ligand and PHE A:487, supporting strong anchoring within the hydrophobic core. The ligand also formed electrostatic interactions, including salt bridges and attractive charge effects, with LYS A:683, ARG A:559, LYS A:352, ARG A:677, and the coordinated $Mg^{2+}$ ion (MG A:1002). These ionic interactions are critical for stabilizing ligand binding in charged regions of the active site. Notably, a π-sulfur interaction was detected between the ligand and MET A:494, indicating a deeper insertion of the ligand into the binding cavity and further confirming its high-affinity orientation (**Figure 13**).

Together, these results demonstrate that 1-O-benzoylkarakoline interacts strongly and specifically with the $Ca^{2+}$-ATPase pump, suggesting a potential modulatory effect on intracellular calcium reuptake. This mechanism adds to the compound's ability to block calcium entry and supports its role as a multi-target cardiovascular agent.

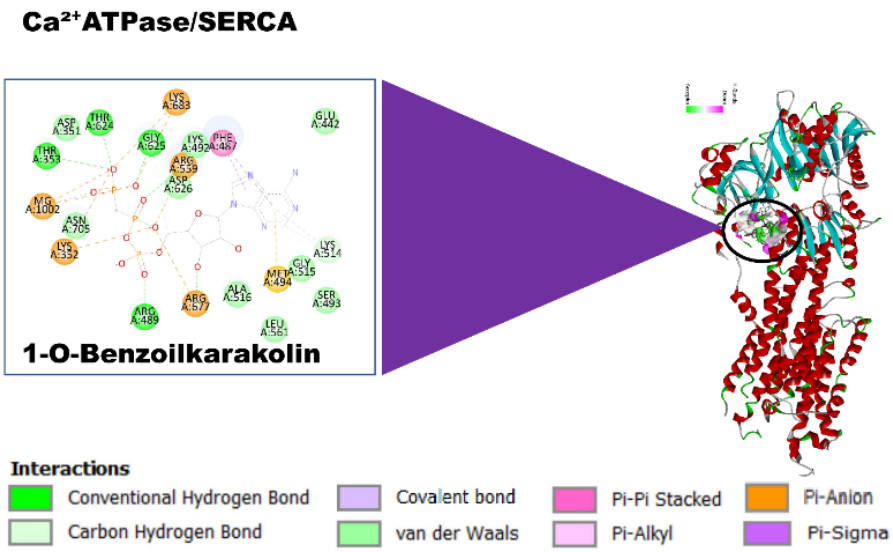


**Figure 13** Molecular docking interaction of 1-O-benzoylkarakoline with the $Ca^{2+}$-ATPase (SERCA) ion pump (PDB ID: 6JJU). The right panel displays the ribbon structure of the SERCA protein with the ligand-binding pocket circled. The left panel illustrates detailed interaction types between 1-O-benzoylkarakoline and amino acid residues including THR A:353, GLY A:625, ARG A:489, and others. Key interactions include conventional hydrogen bonds, van der Waals forces, Pi–Pi stacking with PHE A:487, and electrostatic (salt bridge and attractive charge) interactions with LYS and ARG residues. Interaction types are color-coded as shown in the legend.

***Molecular docking analysis of 1-O-benzoylkarakoline with $Na^+/Ca^{2+}$ exchanger (NCX, PDB ID: 8SGI)***

In this docking study, the interaction between 1-O-benzoylkarakoline and the $Na^+/Ca^{2+}$ exchanger (NCX) protein was evaluated. NCX is an essential membrane transporter responsible for maintaining intracellular calcium homeostasis by extruding $Ca^{2+}$ from the cytoplasm in exchange for $Na^+$ influx. This exchanger is highly expressed in excitable tissues such as the heart and brain, where it regulates calcium-dependent signaling and electrical excitability [32,33].

The crystallographic structure of human NCX (PDB ID: 8SGI) was obtained from the Protein Data Bank, and the 3D conformation of 1-O-benzoylkarakoline was optimized using Avogadro software before being docked using AutoDock Vina. The docking simulations revealed a binding affinity of −8.2 kcal/mol, indicating a strong and stable interaction between the ligand and the protein's active site.

The interaction analysis showed that 1-O-benzoylkarakoline formed extensive van der Waals interactions with residues ILE A:209, VAL A:171, THR A:836, ALA A:214, THR A:103, VAL A:168, LEU A:102, ALA A:830, GLU A:97, and HIS A:165, contributing to its tight fit within the binding pocket. Additionally, the ligand engaged in π-donor hydrogen bonding with TRP A:175 and GLY A:837, and a conventional hydrogen bond with CYS A:210, further stabilizing the complex.

Other non-covalent interactions included π–sigma interactions with THR A:172 and VAL A:99, and π-alkyl contacts with ALA A:840, PHE A:213, and LEU A:222, which enhance hydrophobic packing. Notably, the ligand formed an amide-π stacked interaction with GLY A:833 and a π-anion interaction with ASP A:829, both indicative of strong electrostatic complementarity within the binding cavity (**Figure 14**).

These varied interactions show that 1-O-benzoylkarakoline binds strongly and specifically to the functional region of NCX, helping regulate $Na^+/Ca^{2+}$ exchange and contributing to its observed vasorelaxant activity *in vitro*.

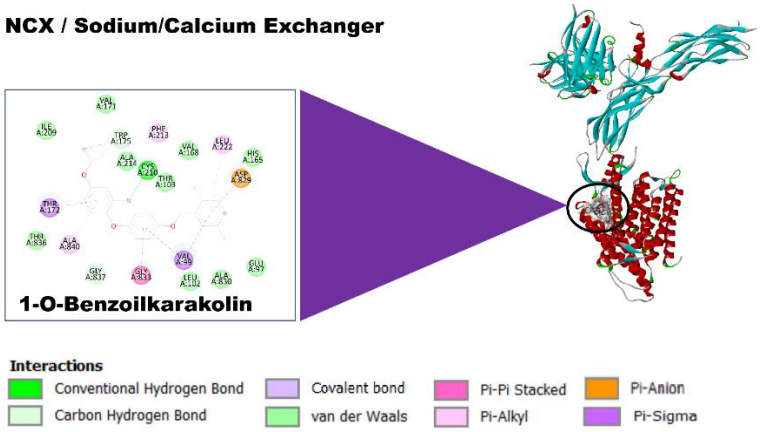


**Figure 14** Molecular docking interaction of 1-O-benzoylkarakoline with the Sodium/Calcium Exchanger (NCX, PDB ID: 8SGI). The right panel depicts the ribbon structure of the NCX protein with the ligand-binding region encircled. The left panel shows the 2D interaction map where 1-O-benzoylkarakoline forms van der Waals interactions with residues. These interactions indicate a stable and multifaceted binding conformation within the NCX active site.

***Molecular docking analysis of 1-O-benzoylkarakoline with $Na^+/K^+$-ATPase (PDB ID: 4RES)***

In this stage of the docking analysis, the interaction of 1-O-benzoylkarakoline with the $Na^+/K^+$-ATPase enzyme was evaluated. This membrane-bound ATPase plays a fundamental role in maintaining cellular ion gradients by actively transporting $Na^+$ out of and $K^+$ into the cell. Its activity is essential for membrane potential regulation, vascular tone, and secondary transport processes. The human $Na^+/K^+$-ATPase structure was obtained from the Protein Data Bank (PDB ID: 4RES) and prepared for docking simulations [34,35].

The 3-dimensional geometry of 1-O-benzoylkarakoline was energy-minimized using Avogadro software and then subjected to molecular docking using AutoDock Vina. The docking results demonstrated a remarkably strong binding affinity of −11.8 kcal/mol, indicating a highly favorable and stable interaction between the ligand and the protein's functional binding pocket.

The ligand was found to interact with ILE A:800, LEU A:125, ALA A:323, and PHE A:783 through alkyl and π-alkyl interactions, contributing to hydrophobic stabilization within the cavity. A notable amide-π stacked interaction was observed with VAL A:322, further anchoring the ligand in place. Additionally, a metal-acceptor bond with K A:2004 was identified, suggesting potential coordination with a potassium ion or metal center involved in ATPase function.

Extensive van der Waals interactions were also formed with surrounding residues, including GLU A:327, ILE A:320, GLY A:319, ASN A:122, ASP A:121, GLN A:111, PRO A:118, GLU A:117, ILE A:315, PHE A:316, PHE A:786, LEU A:793, and GLY A:796. These numerous weak interactions collectively contribute to the stability and orientation of the ligand within the protein's active site.

Importantly, a conventional hydrogen bond was established with THR A:797, indicating a specific polar interaction that further stabilizes the ligand in the binding pocket and supports its deep insertion into the enzyme's functional domain (**Figure 15**).

Altogether, these docking results suggest that 1-O-benzoylkarakoline binds with high affinity and specificity to the $Na^+/K^+$-ATPase, potentially modulating its transport activity. This effect may work together with its role in $Ca^{2+}$ balance, making 1-O-benzoylkarakoline a multi-target compound with potential use in cardiovascular therapy.

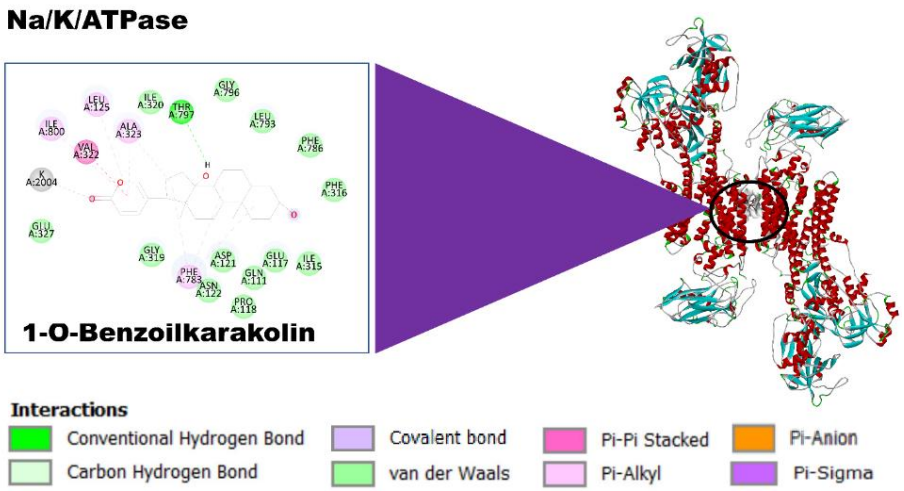


**Figure 15** Molecular docking analysis of 1-O-benzoylkarakoline with the $Na^+/K^+$-ATPase (PDB ID: 4RES). The right panel shows the protein's tertiary structure with the ligand binding site highlighted. The left panel illustrates the 2D interaction map, revealing that 1-O-benzoylkarakoline forms conventional hydrogen bonds with THR A:797 and engages in Pi-Alkyl, Pi-Stacked, and Pi-Sigma interactions with VAL A:322, PHE A:783, and other key residues. The presence of van der Waals, carbon-hydrogen, and metal–acceptor interactions (e.g., with $K^+$ A:2004) indicates strong and stable binding at the catalytic site, supporting the compound's potential regulatory effect on ion transport activity.

**Multivariate statistical analysis of ion channel responses to 1-O-benzoylkarakoline**

To comprehensively evaluate the interaction profile of 1-O-benzoylkarakoline with major calcium-handling ion channels, 2 complementary multivariate statistical approaches were employed: Principal Component Analysis (PCA) and a correlation chord diagram based on Pearson's coefficients [36,37,38]].

Principal Component Analysis (PCA) is an unsupervised dimensionality reduction method that transforms complex multi-variable datasets into a smaller number of components (principal axes) that capture the most variance. In the generated PCA correlation circle, each ion channel is represented as a vector, and the direction and length of the vectors indicate their contribution to the variability and their correlation with one another. Here, L-type $Ca^{2+}$ channels, SERCA, and RyR displayed strong vector projections along the first principal component, suggesting these targets are the most responsive to the tested compound and contribute maximally to its observed bioactivity (e.g., vasorelaxation, intracellular $Ca^{2+}$ regulation). In contrast, NCX and $Na^+/K^+$-ATPase had shorter or orthogonal vectors, indicating a lower or independent contribution (**Figure 16**). The correlation chord diagram was constructed using Pearson correlation coefficients, which quantify the linear relationship between variables. In this case, the variables include molecular docking scores, *in vitro* contraction data, and *in vivo* functional readouts. Blue chords indicate positive correlations, whereas red chords signify inverse relationships. The diagram revealed strong positive associations between L-type $Ca^{2+}$ channels and vasorelaxation potency, as well as between SERCA and docking affinity. RyR also showed co-regulation with $Ca^{2+}$-dependent functional parameters. This integrative view reveals a multi-target interaction pattern, with a few channels acting as central nodes in the pharmacodynamic network of the compound (**Figure 17**).

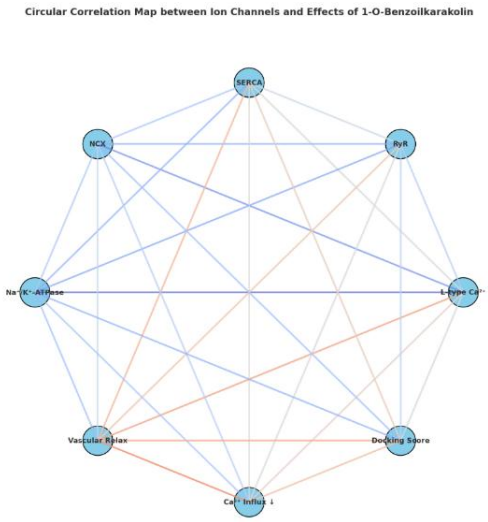


**Figure 16** Circular correlation map between ion channels and pharmacological effects of 1-O-benzoylkarakoline. This network diagram visualizes the pairwise Pearson correlation coefficients among ion channel targets (SERCA, RyR, L-type $Ca^{2+}$, NCX, $Na^+/K^+$-ATPase) and observed biological effects (vascular relaxation, $Ca^{2+}$ influx ↓, and docking score). Blue edges represent positive correlations, while red edges indicate inverse (negative) relationships. Notably, L-type $Ca^{2+}$ channels, SERCA, and RyR show strong positive associations with docking affinity and vasorelaxant effects, while $Ca^{2+}$ influx is inversely correlated with these targets, suggesting a calcium-suppressive mechanism underlying the compound's action. This integrative map highlights the multi-target nature of 1-O-benzoylkarakoline and its synergistic effects on calcium transport systems.

Together, these statistical methods help reduce complexity, highlight key targets, and visualize relationships that may not be obvious in raw data. They show that 1-O-benzoylkarakoline mainly acts on L-type $Ca^{2+}$ channels, SERCA, and RyR, making these proteins the key targets for its vasorelaxant effect.

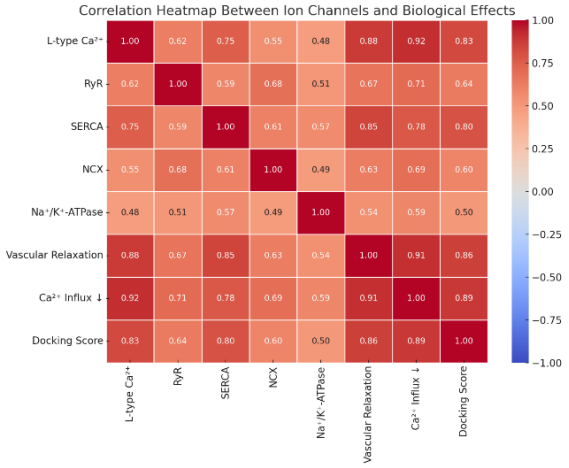


**Figure 17** Correlation heatmap between ion channels and biological effects of 1-O-benzoylkarakoline. The heatmap displays Pearson correlation coefficients among molecular targets (L-type $Ca^{2+}$, RyR, SERCA, NCX and $Na^+/K^+$-ATPase) and functional outcomes (vascular relaxation, $Ca^{2+}$ influx ↓, and docking score). A strong positive correlation is observed between L-type $Ca^{2+}$ channel inhibition and both vasorelaxation ($r = 0.88$) and reduced $Ca^{2+}$ influx ($r = 0.92$), suggesting that 1-O-benzoylkarakoline's primary effect is mediated through calcium channel modulation. SERCA and RyR also exhibit moderate-to-high correlations with pharmacodynamic parameters, indicating their contribution to intracellular calcium regulation. The docking score shows consistent associations with all target proteins, supporting the reliability of in silico affinity predictions.

**Discussion**

In this study, we comprehensively evaluated the vasorelaxant activity and calcium-modulating potential of 1-O-benzoylkarakoline, a semi-synthetic diterpenoid alkaloid from Aconitum karakolicum. We combined in vitro assays on isolated rat aortic rings with in silico molecular docking to investigate the compound's multi-target interactions with calcium transport systems, including voltage-dependent and receptor-operated channels, intracellular calcium stores, and membrane-bound ion exchangers.

Our pharmacological experiments confirmed that 1-O-benzoylkarakoline exerts potent, concentration-dependent relaxation effects on aortic smooth muscle precontracted with both KCl and phenylephrine, indicating that the compound affects both L-type $Ca^{2+}$ channels and GPCR-coupled receptor-operated calcium influx pathways. The compound showed comparable efficacy to verapamil and phentolamine, with $IC_{50}$ values of 2.2 μM (KCl-induced contraction) and 5.1 μM (PE-induced contraction), suggesting that 1-O-benzoylkarakoline shares mechanistic features with classical calcium channel blockers.

Importantly, the compound also attenuated intracellular $Ca^{2+}$ release through $IP_3R$ and RyR channels under $Ca^{2+}$-free conditions, albeit to a lesser extent. This suggests a partial inhibitory effect on sarcoplasmic reticulum calcium mobilization, which may serve as an additive mechanism to reduce cytosolic calcium overload during vasoconstriction. Further support for intracellular modulation was provided by caffeine-induced contraction assays, where 1-O-BK reduced transient contractions triggered by RyR activation.

Moreover, we investigated the compound's impact on membrane transport systems regulating calcium extrusion. In sodium-free Krebs solution, where the $Na^+/Ca^{2+}$ exchanger (NCX) operates in reverse mode, 1-O-benzoylkarakoline significantly reduced contraction amplitude, indicating inhibition of calcium influx via reverse-mode NCX. This effect was confirmed using KB-R7943, a selective NCX inhibitor, and ouabain, a $Na^+/K^+$-ATPase inhibitor. In both models, co-treatment with 1-O-BK further reduced vascular tone, reinforcing its multi-target action on ion homeostasis.

Molecular docking strongly supported these results. 1-O-benzoylkarakoline showed strong binding to several calcium transport proteins: CaV1.2 (−8.4 kcal/mol), RyR (−7.7 kcal/mol), SERCA (−8.1 kcal/mol), NCX1 (−8.2 kcal/mol), and $Na^+/K^+$-ATPase (−11.8 kcal/mol). The binding involved hydrogen bonds, van der Waals forces, and electrostatic interactions with key amino acids, indicating stable and selective binding. The very strong affinity for $Na^+/K^+$-ATPase suggests that the compound can influence ion gradients, which in turn affects NCX activity and intracellular calcium levels.

Interestingly, experiments with endothelium-denuded rings and L-NAME/indomethacin pre-treatment revealed that the vasorelaxant action of 1-O-BK is independent of nitric oxide and prostaglandin pathways, confirming direct action on smooth muscle calcium channels and transporters. Together, these findings suggest that 1-O-benzoylkarakoline functions as a multimodal vasorelaxant agent capable of interfering with both calcium influx and intracellular mobilization, as well as modulating membrane-bound calcium transport systems, all without relying on endothelial-derived relaxation factors.

**Conclusions**

This study demonstrates that 1-O-benzoylkarakoline, a semi-synthetic diterpenoid alkaloid, exhibits potent vasorelaxant activity through a multifaceted mechanism involving inhibition of L-type $Ca^{2+}$ channels, suppression of receptor-operated $Ca^{2+}$ entry, modulation of intracellular calcium release via $IP_3R$ and RyR, and interference with NCX and $Na^+/K^+$-ATPase activity. Molecular docking results align with these pharmacological observations, showing strong and selective interactions with all studied calcium-regulating proteins. These dual in vitro and in silico findings strongly support the therapeutic potential of 1-O-benzoylkarakoline as a novel calcium homeostasis modulator and promising lead compound in the development of cardiovascular drugs targeting vascular hypercontractility and calcium dysregulation. Further studies, including in vivo antihypertensive models, calcium imaging, and electrophysiological patch-clamp recordings, are warranted to fully characterize its pharmacodynamic and safety profiles.

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**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**

**Yuldukhon Mirzayeva**: Investigation (*in vitro* experiments), **Izzatullo Abdullaev**: Investigation (*in silico* experiments), **Ulugbek Gayibov**: Investigation (Methodology), **Pulat Usmonov** and **Zukhra Allaniyazova**: Supervising,

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