doi.org/10.36721/PJPS.2026.39.10.279.1

Antihypertensive and vasorelaxant effects of 2-methoxystypandrone mediated via multiple vascular mechanisms

Adil Javed[1], Atif Ali Khan Khalil[2], Taseer Ahmad[3], Mi-Jeong Ahn[4], Muhammad Ikram[5] and Abdul Jabbar Shah[1]*

[1]Cardiovascular Pharmacology Research Group, Department of Pharmacy, COMSATS University Islamabad, Abbottabad Campus, KPK, Pakistan
[2]Department of Biotechnology, Yeungnam University, Gyeongsan, Republic of Korea
[3]Laboratory of Cardiovascular Research and Integrative Pharmacology, College of Pharmacy, University of Sargodha, Sargodha, Pakistan
[4]College of Pharmacy and Research Institute of Pharmaceutical Sciences, Gyeongsang National University, Jinju, Republic of Korea
[5]Department of Pharmacy, COMSATS University Islamabad, Abbottabad Campus, KPK, Pakistan

Abstract: **Background**: 2-Methoxystypandrone (2-MS), a naphthoquinone from *Reynoutria japonica* Houtt., has diverse pharmacological activities. However, its antihypertensive and vascular effects remain unexplored. **Objectives**: This study investigated the antihypertensive and vasorelaxant potential of 2-MS. **Methods**: I*n vivo*, *invasive* and *in vitro* approaches were employed to study the antihypertensive potential of 2-MS in normotensive and high salt-induced hypertensive rats. The responses were recorded and analyzed using the PowerLab Data Acquisition system. *In-silico* docking predicted potential cardiovascular targets and binding interactions. **Results**: Intraperitoneal administration of 2-MS (1,3 and 5 mg/kg/day) for 28 days significantly prevented the rise in mean arterial pressure (MAP) and heart rate in hypertensive rats. Intravenous administration in normotensive rats produced a dose-dependent fall in MAP. Pretreatment with $_{L}$-NAME and atropine attenuated the hypotensive response, suggesting involvement of the endothelial nitric oxide (NO)-linked muscarinic pathway, whereas pretreatment with indomethacin did not alter it. *In-vitro*, 2-MS relaxed the aortic rings precontracted with phenylephrine. This relaxation to 2-MS was abolished with endothelial removal or $_{L}$-NAME and atropine pretreatment. 2-MS inhibited both voltage- and receptor-operated Ca^{2+} channels and activated TEA- and 4-minopyridine–sensitive K^{+} channels. Molecular docking revealed strong affinity towards muscarinic M_2 receptors, supporting endothelial and smooth muscle actions. **Conclusion**: These findings demonstrate that the antihypertensive and vasorelaxant effects of 2-MS are mediated by both endothelium-dependent and endothelium-independent pathways.

Keywords: Calcium channels; Hypertension; Nitric oxide (NO); Potassium channels; 2-Methoxystypandrone

Submitted on 12-10-2025 – Revised on 12-11-2025 – Accepted on 11-12-2025

INTRODUCTION

Phenolic compounds represent one of the most diverse classes of secondary metabolites found abundantly in the plant kingdom. They have long been known for diverse biological activities, most notably their anti-inflammatory, antioxidant and cardiovascular protective activities (Shahidi and Ambigaipalan, 2015). These compounds contain one or more hydroxyl groups attached to the aromatic rings at different positions, a feature that enables them to regulate endothelial function and oxidative stress. Both these factors are significantly implicated in the onset and progression of hypertension (Rodrigo *et al*., 2011; Virdis and Taddei, 2016). Many phenolic compounds, such as quercetin, curcumin, and resveratrol, lower blood pressure through different mechanisms, including enhancing nitric oxide (NO) production, suppressing the renin–angiotensin system, and reducing vascular inflammation (Touyz and Briones, 2011).

Among the phenolic subclasses, naphthoquinones have attracted considerable attention owing to their distinct redox properties and ability to interact with various cellular targets (Babula *et al*., 2009; Pereyra *et al*., 2019). Naphthoquinones such as juglone and plumbagin have numerous reported activities like antimicrobial, anticancer and anti-inflammatory effects, mainly due to their quinone structure which can halt redox cycling and the formation of reactive oxygen species (Tandon and Kumar, 2013).

2-Methoxystypandrone (2-methoxy-6-acetyl-7-methyljuglone) (Fig. 1), a naturally occurring naphthoquinone from *Reynoutria japonica* Houtt. is gaining widespread recognition due to its potent pharmacological profile. It has previously been studied for its cytotoxic and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB)- inhibitory activities (Mesalam *et al*., 2017). 2-Methoxystypandrone has demonstrated potent anti-inflammatory effects, primarily by inhibiting Signal Transducer and Activator of Transcription 3 (STAT3) signaling. It blocks Janus kinase 2 (JAK2) and prevents STAT3 phosphorylation. Since

**Corresponding author:* e-mail: jabbarshah@cuiatd.edu.pk

STAT3 promotes vascular inflammation and remodeling in hypertension, its inhibition offers cardiovascular protection. 2-Methoxystypandrone also inhibits IKKβ, a kinase vital for the activation of NF-κB, thereby reducing the transcription of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) (Kuang *et al*., 2014). By blocking these signaling pathways, 2-methoxystypandrone helps prevent inflammation that leads to endothelial dysfunction and vascular damage.

Despite these pharmacological findings, its potential role in cardiovascular disorders, especially hypertension, remains unexplored. Considering its phenolic nature and mechanistic similarity to known vasoprotective agents, 2-methoxystypandrone might provide benefits in vascular dysfunction and high blood pressure through multiple mechanisms, including antioxidant, anti-inflammatory and endothelial-modulating actions.

In this study, we explored the antihypertensive potential of 2-methoxystypandrone and its underlying mechanism(s) using *in vivo* and *in vitro* rat model.

MATERIALS AND METHODS

Chemicals and standards

All the chemicals and reagents utilized in this study were of analytical grade and high purity. Acetylcholine chloride, atropine sulfate, heparin sodium, N^G-nitro-L-arginine methyl ester ($_L$-NAME), norepinephrine bitartrate, phenylephrine hydrochloride (PE) and verapamil hydrochloride were procured from Sigma-Aldrich (St. Louis, MO, USA). The chemical constituents of Krebs' physiological solution included calcium chloride, D-glucose, magnesium sulfate, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate and sodium chloride (Merck-Schuchardt, Muenchen, Germany), along with ethylene glycol tetraacetic acid, which was obtained from Sigma-Aldrich.

Two percent dimethyl sulfoxide (DMSO) in distilled water was used for the preparation of stock solutions and all dilutions were prepared fresh just before the experimentation. 2-Methoxystypandrone was isolated from the roots of *Reynoutria japonica* Houtt. by Dr. Atif Khan Khalil and Mi-Jeong Ahn. The structure of 2-methoxystypandrone was confirmed using mass spectrometry, ^{1}H NMR and ^{13}C NMR, while purity was confirmed using High Performance Liquid Chromatography (HPLC) technique (Figs. S1-S4).

Experimental animals and housing conditions

In the present study, Sprague-Dawley (SD) rats representing both sexes and weighing 200–250 g were recruited for *in-vitro* and *in-vivo* experimentation. Rats were kept in the animal facility at COMSATS University Islamabad (CUI), Abbottabad campus, under standard laboratory conditions in accordance with the *Guidelines for the Care and Use of Laboratory Animals* (National Research Council, 1986). The experimental protocols were reviewed and approved by the Research Ethics Committee at COMSATS University Islamabad (CUI), Abbottabad Campus (Approval No. PHM.Eth/CS-M02-07-2975). Throughout the study period, rats had unrestricted access to standard pellet food and tap water.

Determination of mean arterial pressure (MAP)

The procedures described by Lawler *et al*. (1987) and Vasdev *et al*. (2003) were adopted with slight amendments. SD rats, preferably male and weighing between 200 and 250 g, were given 8% NaCl in water and diet for 28 days. After this period, rats with systolic blood pressure between 140–190 mmHg were considered as hypertensive (Ahmad *et al*., 2020). Two different protocols were used. In the first protocol, rats were randomly assigned to five groups with n=5 each. Group 1 served as the normotensive control group and received normal saline. Group 2 was given 8% NaCl diet and water and served as the hypertensive control. Groups 3, 4 and 5 received 8% NaCl diet along with intraperitoneal administration of 2-MS (1,3 and 5 mg/kg), respectively. The doses of 1, 3 and 5 mg/kg were selected based on preliminary pilot experiments conducted in our laboratory, where we observed that these doses produced measurable pharmacological effects without inducing overt toxicity.

In the second protocol, Group 1 (n=5) was a normal control fed with normal saline, while Group 2 (n=5) was fed water and an 8% NaCl diet for 28 days. After 28 days, the effect of the test compound on MAP was assessed through systolic and diastolic blood pressure measurements.

Experimental protocol

Male SD rats were subjected to anesthesia with intraperitoneal injections of xylazine (20 mg/kg) and ketamine (100 mg/kg). The trachea, along with the carotid artery and the right jugular vein, was carefully exposed through surgical incision to allow cannula insertion. Polyethylene (PE)-20 tubing was used to cannulate the trachea to maintain respiration and PE-50 tubing to cannulate the carotid artery and right jugular vein to detect and record arterial pressure and to inject intravenous drugs, respectively. Arterial blood pressure was measured using a pressure transducer (MLT 0201) connected to the PowerLab (ML 846, ADInstruments, Australia) Data Acquisition System. Heparinized saline (0.1 mL) was injected as needed to avoid coagulation and body temperature was sustained using an overhead heat source. Rats were allowed a 20–30-minute stabilization period prior to experimentation. Baseline systolic and diastolic blood pressures were recorded following the administration of norepinephrine and acetylcholine at the doses of 1 μg/kg, each flushed with 0.1 mL saline (Salma *et al*. 2018; Van Vliet and Montani 2008).

In the first protocol, 2-methoxystypandrone (2-MS) was administered intraperitoneally for 28 consecutive days and blood pressure was recorded for 30 minutes in each group. In the second protocol, 2-MS was administered intravenously at different doses (0.1 mL of distilled water as vehicle). After each dose, blood pressure was permitted to stabilize to its pre-treatment value before subsequent administration. Variations in systolic and diastolic pressures were employed to compute the mean arterial pressure (MAP) using the following formula:

$$\text{MAP} = Diastolic\ BP \times \frac{1}{3}(Systolic\ BP - Diastolic\ BP)$$

The percentage reduction in MAP was then calculated as:

$$\%\ \text{Fall} = \frac{Control - Fall}{Control} \times 100$$

The effect of 2-MS on blood pressure was assessed both alone and in the presence of 2 mg/kg atropine, 20 mg/kg $_L$-NAME and 10 mg/kg indomethacin *in-vivo*. Rats were injected with standard drugs for approximately 10-15 minutes before testing 2-MS. After treatment, different doses of 2-MS were injected and the effects on systolic and diastolic blood pressures were recorded and compared (Shah and Gilani, 2009; Shah *et al*., 2014).

Vascular reactivity studies

Isolated rat aortic ring preparations

Isolated aortic tension experiments were performed following the method of Furchgott and Zawadzki (1980) with appropriate amendments. From normal SD rats, the aorta was carefully dissected to ensure the integrity of the endothelium. Adherent connective and adipose tissues were gently cleaned; after that, the aorta was carefully segmented into 2–3 mm-long rings and individually suspended in a 10 mL organ bath enriched with Krebs' solution, continuously gassed with carbogen (95% O_2 , 5% CO_2), and kept at 37°C. A basal tension of 2 g was standardized and the tissues were stabilized for 30–45 mins before initiating the experimental protocol.

Effect of 2-MS on phenylephrine- and K^+ (80 mM)-induced contractions

The protocol employed was adapted from previously adopted methodologies (Chan *et al*., 2006; Qamar *et al*., 2018) incorporating slight procedural adjustments. Isolated aortic rings were precontracted using 1 µM phenylephrine and 80 mM potassium chloride, which serve as standard vasoconstrictors, until a stable plateau response was achieved. Cumulative dosing of 2-methoxystypandrone was applied to establish concentration-dependent response curves and the degree of relaxation was quantified relative to the percentage of the initial contraction produced by the agonist. In certain preparations, Mechanical denudation of the endothelium layer was performed by delicately scraping the inner surface of the vessel with fine forceps. The effectiveness of endothelial removal was confirmed when the tissues showed less than 80% relaxation in response to acetylcholine (0.1 µM).

Effect of $_L$-NAME, atropine and indomethacin on 2-MS-induced vasorelaxation

To elucidate the mechanisms contributing to endothelium-dependent vasorelaxation, aortic rings with intact endothelium were preincubated with specific inhibitors, including $_L$-NAME (10 µM), indomethacin (1 µM) and atropine (1 µM) for 15–20 min. Under these conditions, cumulative concentrations of 2-MS were administered to phenylephrine (1 µM)-precontracted aortic rings. The resulting concentration–response curves (CRCs) were recorded and the vasorelaxant responses were compared across the treatment conditions.

Effect of 2-MS on intracellular Ca^{2+} stores

2-MS-induced vascular reactivity was evaluated for its effects on calcium mobilization via calcium release from intracellular stores and through receptor-operated channels (ROCs) in isolated rat aortic rings. Following an initial contraction induced by 1 µM phenylephrine in standard Krebs' solution, the aortic rings were subsequently immersed in Ca^{2+} -free Krebs solution containing EGTA and incubated for 15 minutes before re-exposure to phenylephrine (1 µM). In the experimental group, rings were preincubated with 2-MS (1-100 µg/mL) for 30 minutes before applying phenylephrine (1 µM). Subsequently, tissues were rinsed in triplicate with standard Krebs' solution and equilibrated for at least 40 minutes to replenish intracellular Ca^{2+} stores. The incubation medium was then refreshed again with Ca^{2+} -free Kreb's solution and equilibrated for 15 minutes before a second phenylephrine-induced contraction (1 µM) was recorded in the presence of 2-MS, which was added 30 minutes earlier. Comparative analysis was performed between the two contractions. The same method was performed using verapamil (0.001–10 µg/mL) as the reference calcium channel blocker.

Effect of 2-MS on voltage-operated Ca^{2+} channels

The effects of 2-MS on voltage-dependent Ca^{2+} channels (VDCs) were evaluated and compared with those of the standard calcium channel blocker, verapamil. For this purpose, the bath solution was substituted with Ca^{2+} -free EGTA-enriched Krebs' medium to remove extracellular calcium. Subsequently, standard concentration–response curves (CRCs) for $CaCl_2$ (0.01–10.0 mM) were constructed to establish baseline responses. To assess calcium channel blockade, aortic rings were preincubated with 2-MS (30–100 µg/mL) for 30 minutes and $CaCl_2$ -induced contractions were recorded. The same protocol was repeated with verapamil to establish reference CRCs for calcium channel inhibition (Javed *et al*., 2024).

Effect of 2-MS on phenylephrine-induced contractions in the absence and presence of k^+-channel blockers

To assess the involvement of potassium channels in the vasorelaxant activity of 2-MS, phenylephrine-induced contractions were recorded in both control conditions and in the presence of specific potassium channel blockers. The

blockers included 4-aminopyridine (4-AP, 1 mM), barium chloride ($BaCl_2$, 1 mM), tetraethylammonium (TEA, 5 mM) and glibenclamide (10 µM), each tested in separate experimental preparations. The blockers were added 20 minutes before the phenylephrine administration. After sustained phenylephrine contraction, cumulative concentrations of 2-MS were applied to construct relaxation response curves (Ahmad *et al*., 2022).

In-silico docking studies

In-silico strategy was employed to assess the inhibitory potential of 2-MS at the molecular level. Molecular docking was performed using AutoDock (version 1.5.7) against selected cardiovascular targets. The crystal structures of muscarinic M_2 and M_3 receptors, β_1- and β_2-adrenergic receptors, angiotensin II receptor and angiotensin-converting enzyme (ACE) were sourced from the repository of the Protein Data Bank with PDB IDs 4MQT, 4DAJ, 2R4R, 4ZUD and 1O8A, respectively. Both the protein structures and the ligand were prepared by protonation and energy minimization, following established protocols. Post-docking, the resulting conformations were evaluated based on binding affinities and key molecular interactions within the active sites using Discovery Studio Visualizer (Heo *et al*., 2023; Drabek *et al*., 2025).

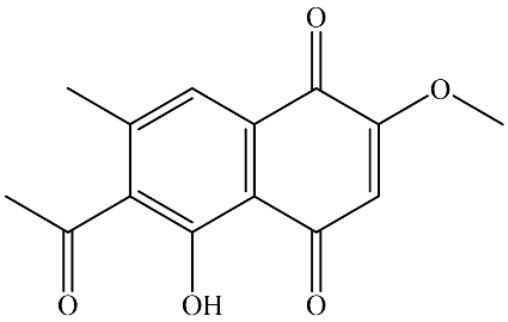

Fig. 1: Structure of 2-methoxystypandrone.

Statistical analysis

All experimental data were presented as mean ± SEM. The median effective concentration (EC_{50}) values were calculated together with their corresponding 95% confidence intervals (CI). Statistical significance among groups was determined using either one-way analysis of variance (ANOVA) followed by Dunnett's post hoc multiple comparison test or Student's *t*-test, as required. Statistical analyses were performed with GraphPad Prism software (version 8; GraphPad Software, San Diego, CA, USA). The probability value of $p<0.05$ was regarded as statistically significant, with significance level denoted as *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

RESULTS

Effect of 2-MS on mean arterial pressure (MAP)

The control MAP values were measured in both normotensive and hypertensive rats, with a significant ($p<0.001$) increase in hypertensive rats (Fig. 2A). Intraperitoneal treatment with 2-MS (1, 3 and 5 mg/kg) for consecutive 28 days significantly ($p<0.001$) prevented the development of hypertension in the experimental groups. The MAP recorded was 117.7, 99.6 and 83.82 mmHg (Fig. 2A), respectively, compared to the hypertensive control which was 151.1 mmHg and the verapamil treated group (10 mg/kg/day) at 79.90 ± 2.32 mmHg (Fig. 2A). Additionally, 2-MS at 1, 3 and 5 mg/kg significantly ($p<0.001$) decreased heart rate (beats per minute), with values of 331.4 ± 9.91, 306.4 ± 8.99 and 247.8 ± 5.04, respectively, when compared to the hypertensive control (385.2 ± 6.26) and the verapamil-treated group (279.8 ± 9.68) (Fig. 2B).

Effect on MAP after intravenous administration of 2-MS

Intravenous injection of norepinephrine (1 µg/kg) and acetylcholine (1 µg/kg) caused a rise (82.75%) and a fall (56.5%), respectively, in mean arterial pressure (MAP) (Fig. 2C), thus confirming the protocol. 2-MS resulted in a dose-dependent decrease in MAP in normotensive anesthetized rats following intravenous administration. The percent fall in MAP at different doses (0.01-1 µg/kg) was 16.13±0.75, 23.80±1.27, 32.93±1.26, 39.16±1.98 and 44.83±1.89 mmHg, respectively (Figs. 2D-E).

Effect of intravenous 2-MS on MAP in normotensive rats pretreated with $_L$-NAME, atropine and indomethacin

This experiment was performed in anesthetized normotensive rats pretreated with specific pharmacological blockers to elucidate the mechanisms underlying the blood pressure-lowering effect of 2-MS. The rats received pretreatment with $_L$-NAME (20 mg/kg), atropine (2 mg/kg) and indomethacin (10 mg/kg) prior to intravenous administration of 2-MS. In the $_L$-NAME pretreated rats, the percent fall in MAP induced by 2-MS was 8.8 ± 0.21, 11.46 ± 0.43, 16.93 ± 1.08, 20.50 ± 0.85 and 25.20 ± 0.93, respectively (Fig. 2E). Similarly, the percent fall in MAP induced by 2-MS in atropine-pretreated rats was 4.93 ± 0.72, 9.10 ± 0.56, 14.03 ± 1.67, 16.27 ± 1.73 and 19.20 ± 0.6 mmHg, respectively (Fig. 2E). Additionally, in the indomethacin-pretreated rats, the percent decrease in MAP was 15.1 ± 0.34, 21.43 ± 1.11, 32.47 ± 1.41, 37.9 ± 1.17 and 43.23 ± 1.28 mmHg, respectively (Fig. 2E).

Vascular reactivity studies

Effect of 2-MS on vascular tone

Thoracic aortae from rats in normal control, hypertensive control and 2-MS treated groups (1, 1,3 and 5 mg/kg) were isolated for vascular dysfunction studies. Isolated rat aorta from the normal control, precontracted with phenylephrine, showed relaxation to acetylcholine with an EC_{50} value of 1.09 µg/mL (0.93-1.27) (Fig. 3). Rat aortic rings from hypertensive control exhibited less than 10% relaxation at 10 µM concentration of acetylcholine, compared to 100% relaxation in the normal control group (Fig. 3).

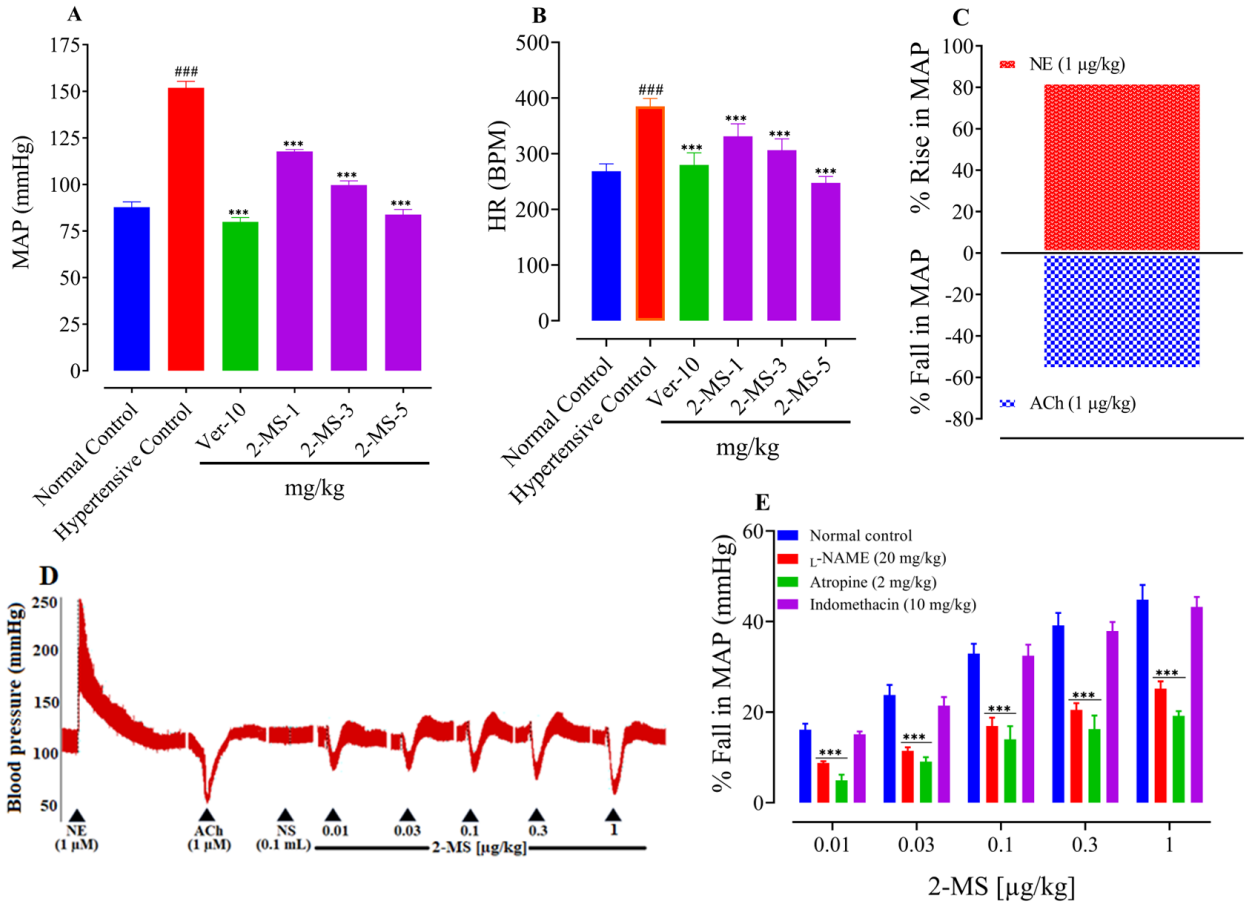

Fig. 2: Graphs showing the effects on (A) mean arterial pressure (MAP) and (B) heart rate (HR) after 28 days of intraperitoneal (i.p.) treatment with verapamil (Ver-10 mg/kg) and 2-MS (1,3 and 5 mg/kg) in high salt-induced hypertensive rats; (C) displays the percent rise and fall in mean arterial pressure in normotensive rats under anesthesia; (D) presents a typical tracing showing the effects of norepinephrine (NE), acetylcholine (ACh) and 2-MS on blood pressure; (E) illustrates the effect of 2-MS on MAP in normotensive anesthetized rats and compares the percentage fall in MAP caused by 2-MS in normotensive rats pretreated with L-NAME (20 mg/kg), atropine (2 mg/kg) and indomethacin (10 mg/kg). Data are expressed as mean ± SEM (n=5). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test. ***p<0.001 indicates a significant difference compared to hypertensive control and ###p< 0.001 vs normal control.

Isolated aortic rings from the 1 mg/kg 2-MS treated group demonstrated partial vasorelaxant response to acetylcholine, with 36% relaxation (Fig. 3). In the 3 mg/kg 2-MS treated group, a further enhanced response to acetylcholine was observed, with a maximum relaxation of 43%. Aortic rings from the 5 mg/kg 2-MS treated group exhibited the highest vasorelaxant response to acetylcholine among the three groups, with 80% relaxation and an EC_{50} value of 3.29 µg/mL (2.51-4.33) (Fig. 3).

Effect of 2-MS on phenylephrine and K^+ (80 mM) precontractions

2-Methoxystypandrone (2-MS) relaxed the rats' aortic rings in a concentration-dependent manner, precontracted with phenylephrine (1 µM) and high-potassium (80 mM) solutions. The EC_{50} values were 13.84 µg/mL (11.24–16.86) and 62.79 µg/mL (59.03–66.75), respectively, compared with the standard calcium channel blocker verapamil, as shown in figure. 4.

Endothelium-dependent and -independent effects

Rat aortic rings with intact endothelium were precontracted with phenylephrine (1 µM). Cumulative addition of 2-MS elicited concentration-dependent vasorelaxation with an EC_{50} value of 13.84 µg/mL (11.24-16.86) (Fig. 5A). The removal of endothelium markedly attenuated this response, with an EC_{50} value of 31.23 µg/mL (Fig. 5A), confirming the involvement of endothelium-derived relaxing factors. When intact aortic rings were preincubated with L-NAME (10 µM), it completely inhibited the vasorelaxant response induced by 2-MS and elicited a pronounced vasoconstriction (Fig. 5A), indicating a crucial role of NO.

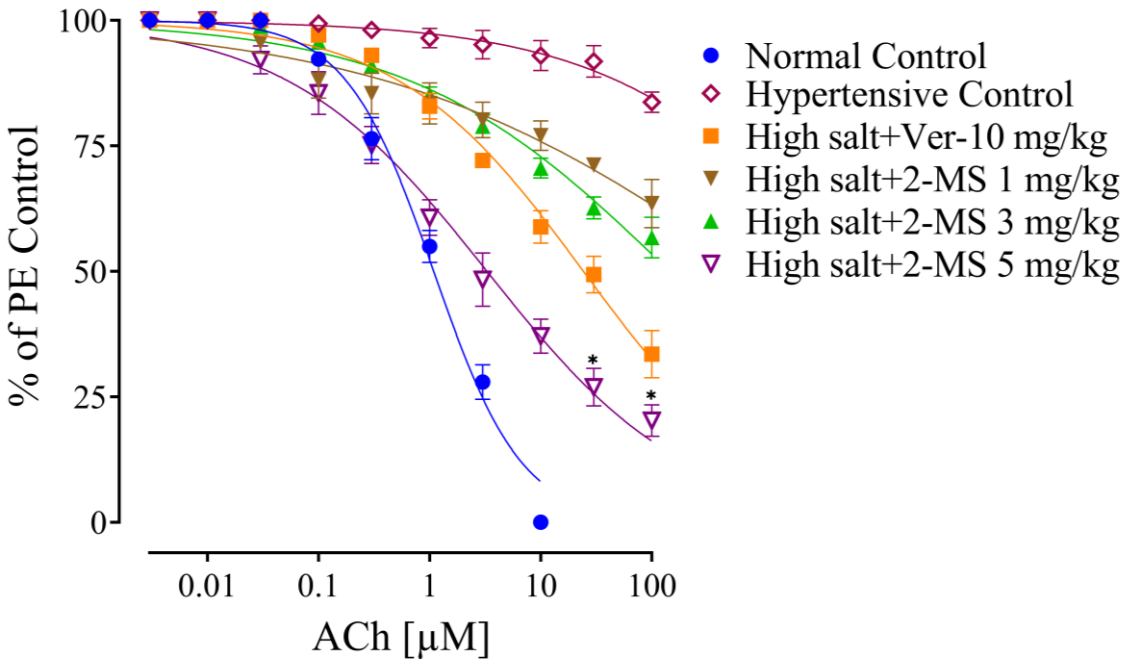

Fig. 3: Graph showing the vasorelaxant response of acetylcholine (ACh) on phenylephrine (PE)-induced contractions in isolated rat aortic rings from normal control, hypertensive control, verapamil (10 mg/kg treated rats), and 2-MS treated groups (1,3, and 5 mg/kg). Values are expressed as mean ± SEM (n=4-5). One-way ANOVA followed by post hoc Dunnett's multiple comparison test was used to perform the statistical analysis. *p<0.05 indicates a significant difference compared to the hypertensive control.

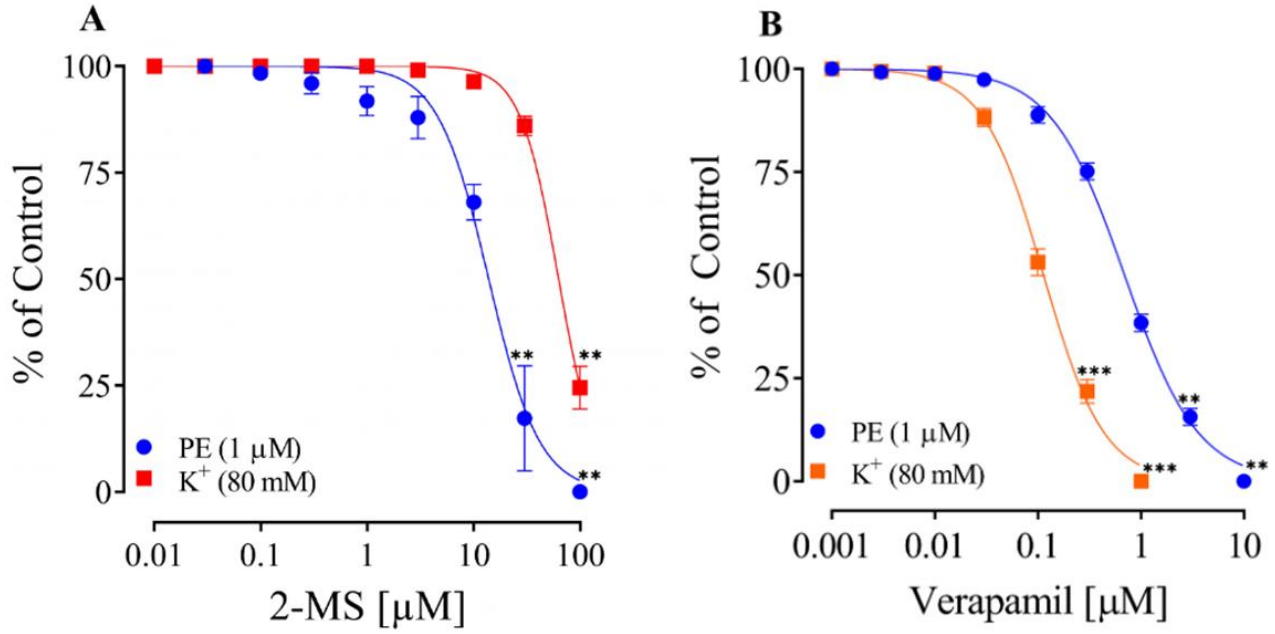

Fig. 4: Graphs illustrating the vasorelaxant effect of (A) 2-MS and (B) verapamil on precontractions induced by phenylephrine (PE) and K^+ (80 mM) in rat aortic preparations. Values are expressed as mean ± SEM (n=5-7). One-way ANOVA followed by post hoc Dunnett's multiple comparison test was used to perform the statistical analysis. Where **p<0.01, ***p<0.001, indicates statistically significance difference.

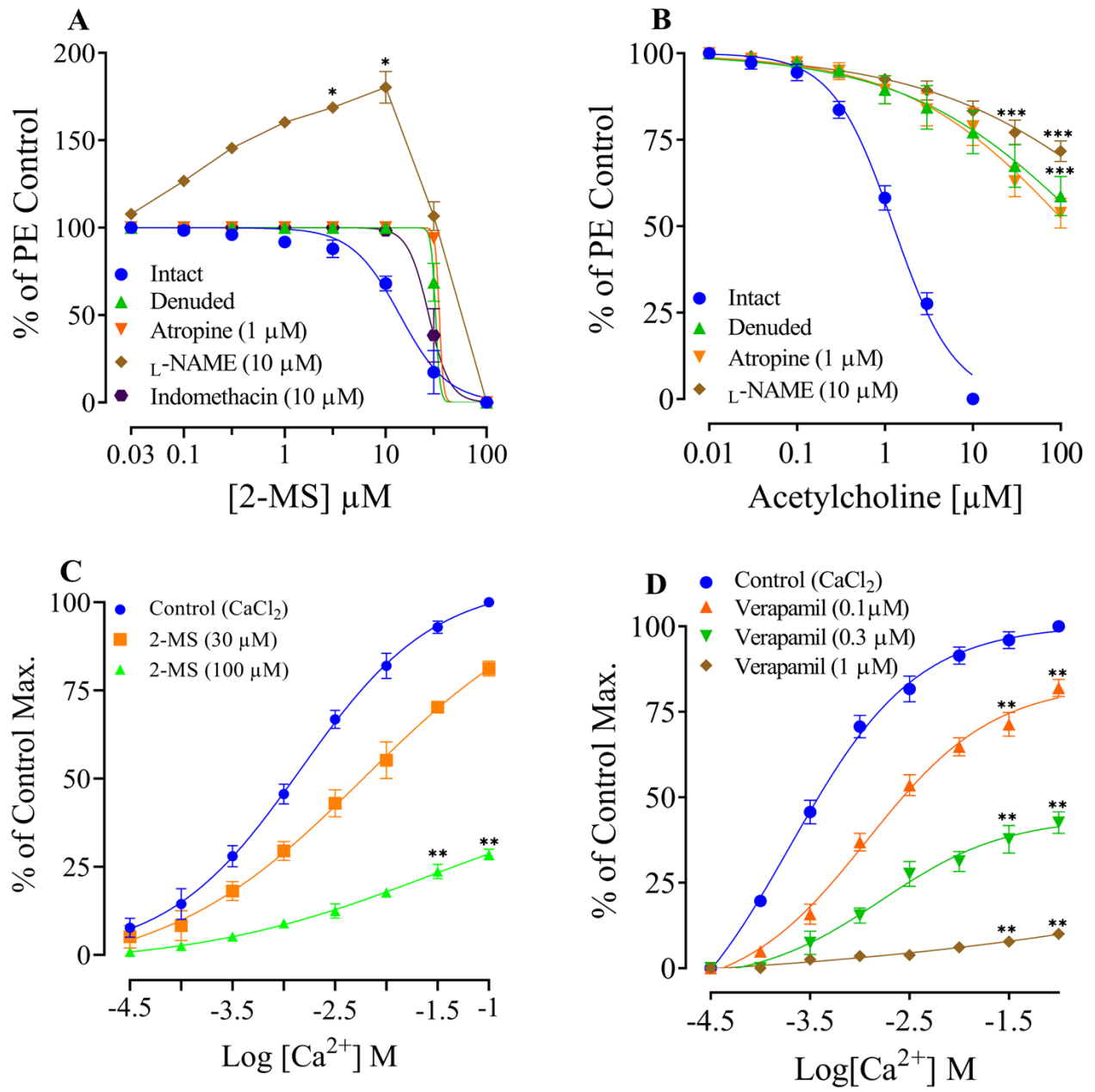

Fig. 5: Graphs displaying the vasorelaxant response of (A) 2-MS and (B) acetylcholine on phenylephrine-pre-contracted rings in intact, denuded, and pretreated rat aortic rings; also, the inhibitory effects of (C) 2-MS and (D) verapamil on Ca^{2+}-concentration response curves (CRCs) in isolated rat aorta preparations, constructed in Ca^{2+}-free/EGTA medium. Values are presented as mean ± SEM (n=5-7). Statistical analysis was performed using one-way ANOVA followed by post hoc Dunnett's multiple comparison test. Where *p<0.05, **p<0.01, and ***p<0.001 indicate significant differences.

Furthermore, pretreatment with atropine (1 µM), a muscarinic receptor antagonist, significantly reduced the vasorelaxant activity of 2-MS, with an EC50 of 39.0 µg/mL, suggesting partial mediation via muscarinic receptor-linked NO release. On the contrary, incubation with indomethacin (10 µM) moderately shifted the concentration-response curve to the right, yielding an EC50 of 26.78 µg/mL (Fig. 5A), indicating a minor contribution of prostacyclin-dependent vasorelaxation. Similar protocols were also used to assess the vasorelaxant response to acetylcholine (Fig. 5B).

Effect of 2-MS on voltage-dependent calcium channels

Preincubation with 2-MS (30-100 µg/mL) attenuated the $CaCl_2$ (0.01-1 mM)-induced contractions in a Ca^{2+}-free/EGTA Krebs' solution, shifting the Ca^{2+} concentration response curves rightwards with a reduction in the maximal contractile response (Fig. 5C), similar to verapamil (Fig. 5D).

Effect of 2-MS on intracellular calcium stores

In one series of experiments, the influence of 2-MS on phenylephrine (1 μM)-induced transient contractions was evaluated. Rat aortic rings, when preincubated with 2-MS (1–100 μg/mL), significantly inhibited the phenylephrine-induced contractile response in Ca^{2+}-free/EGTA Krebs' solution (Figs. 6A-B). A comparable inhibitory pattern was observed with verapamil, suggesting interference with calcium release from intracellular stores (Fig. 6C).

Effect of 2-MS on phenylephrine precontractions in the presence and absence of K^+-channel blockers

To determine whether the vasorelaxant activity of 2-MS involves activation of K^+ channels, selective K^+ channel inhibitors were employed. These included $BaCl_2$ (K_{ir} blocker), 4-aminopyridine (4-AP, voltage-gated K^+ channel blocker), tetraethylammonium (TEA, K_{Ca} channels blocker) and glibenclamide (K_{ATP} sensitive channels blocker). Aortic rings pretreated with 4-aminopyridine and tetraethylammonium significantly reduced the relaxant response to 2-MS, particularly at lower concentrations.

The EC_{50} values for control, $BaCl_2$ (1 mM), 4-AP (1 mM), TEA (5 mM) and glibenclamide (10 μM) were 13.84 (11.24-16.86), 32.92, 87.89 (81.11 to 95.53), 66.78 (64.08 to 69.62) and 31.37 μg/mL, respectively (Fig. 7).

In-silico docking studies

To evaluate the antihypertensive potential of 2-MS *in-silico*, we tested it against various proteins, including muscarinic M_2 receptor (pdb id = 4MQT), muscarinic M_3 receptor (pdb id = 4DAJ), β_1 and β_2 receptor (pdb id = 2r4r), angiotensin-II receptor (pdb id = 4ZUD) and ACE enzyme (pdb id = 1O8A). After docking simulation, the best confirmations were selected (Fig. 8). The results show that 2-MS interacts with M_2 receptor residues TYR104, ILE178 and CYS176 through hydrogen bonding, while with Tyr83, Trp84 through hydrophobic interaction, more specifically pi-pi interaction, with a binding energy of -6.1886 kcal/mol (Fig. 8A; Table 1). On the other side, 2-MS exhibits a hydrogen bonding interaction with residues TYR148 and ASN507, while pi-pi interaction with TYR506 and TRP503 of the muscarinic M_3 receptor, having the binding energy of -5.8962 kcal/mol (Fig. 8B Table 1). Another key target protein is β_1 and β_2 receptors for which inhibition has been explored. 2-MS showed hydrogen binding interaction with core amino acids including LYS42, ASP165 and SER168 with binding energy -4.7605 kcal/mol (Fig. 8C; Table 1). The 2-MS potential was also studied against the angiotensin II receptor. The results show that 2-MS interacts with the important residues TYR87 and ARG167 through hydrogen bonding and exhibits hydrophobic interactions with Trp84 in the active site of the protein, with a binding energy of -5.0721 kcal/mol (Fig. 8D, Table 1). Lastly, 2-MS was docked into the active site of the ACE enzyme. The results show that 2-MS interacts with HIS353, HIS513, LYS454 and TYR520 through hydrogen bonding, while with Tyr523 and HIS383 residues, 2-MS showed hydrophobic interaction with a binding energy of -5.4197 kcal/mol (Fig. 8E, Table 1).

DISCUSSION

The current study investigates the antihypertensive potential of 2-methoxystypandrone (2-MS) in normotensive and high salt-induced hypertensive SD rats. Chronic intraperitoneal administration of 2-MS (1,3 and 5 mg/kg/day) to high salt-induced hypertensive rats for 28 days significantly ($p<0.001$) prevented the rise in MAP. This treatment also reduced heart rate in a dose-dependent manner, with the greatest effect at the highest dose. Acute intravenous administration of 2-MS in normotensive anesthetized rats induced a rapid, dose-dependent fall in MAP, thus validating the *in-vivo* antihypertensive effect. The reduction in MAP after i.v. administration reflects the pharmacological profile of natural polyphenols, which have been previously reported to promote vasodilation via distinct mechanistic pathways (Testai and Calderone, 2017). These findings encouraged us to further investigate the underlying mechanism(s) *in-vivo*.

To delineate the possible contribution of endothelial mediators, involvement of NO was studied. NO is a lipophilic gas, synthesized in the endothelial cells by the enzyme NO synthase. NO synthase converts L-arginine to citrulline and NO, which then diffuses to the vascular smooth muscle cells, causing vasorelaxation (Gonzalez *et al*., 2025). Pretreatment with $_L$-NAME, an inhibitor of NO synthase (Pechanova *et al*., 2020), significantly ($p<0.001$) attenuated (45%) the reduction in MAP. This attenuation in MAP after $_L$-NAME treatment indicates the central role of nitric oxide (NO) in the antihypertensive effect of 2-MS. The release of nitric oxide in blood vessels is linked with muscarinic receptor activation (Moncada and Higgs, 1995). In vascular endothelium, muscarinic receptors, particularly M_3 receptors, stimulate endothelial NO release and hyperpolarization, thereby contributing to vasodilation (Benyo *et al*., 2005). To test if 2-MS interacts with vascular muscarinic receptors, normotensive rats under anesthesia were pretreated with a muscarinic receptor antagonist, atropine (Van Zwieten and Doods 1995). This pretreatment significantly ($p<0.001$) reduced (58%) the effect of 2-MS on MAP, compared to the normal control (45%), highlighting the contribution of muscarinic receptor activation. The results indicate that $_L$-NAME and atropine partially reversed (45% and 58%, respectively) the antihypertensive effect of 2-MS, suggesting that additional vascular mediators may be involved.

In addition to synthesizing NO, the vascular endothelium releases other vasorelaxant mediators, such as prostacyclin (PGI_2), a potent vasodilator (Gryglewski *et al*., 2001).

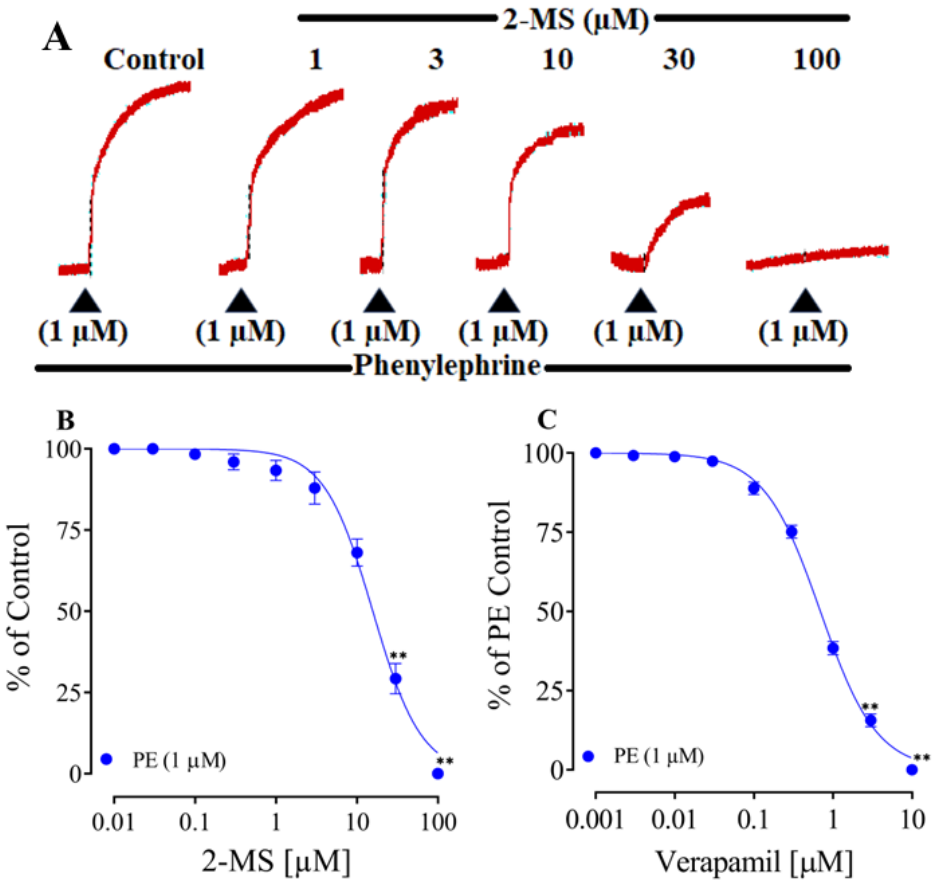

Fig. 6: (A) a typical tracing showing the inhibitory effect of increasing concentrations of (B) 2-MS and (C) verapamil on the initial peak formation of phenylephrine (PE)-induced contractions in Ca^{2+}-free/EGTA medium. One-way ANOVA followed by post hoc Dunnett's multiple comparison test was used to analyze the data statistically. Values are shown as mean ± SEM (n=4-5). **p<0.01 represents the significant difference.

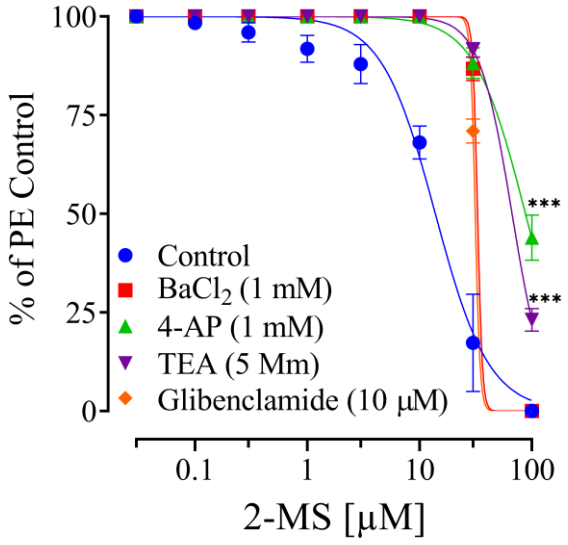

Fig. 7: The response of 2-MS to phenylephrine (1 µM) precontractions in intact and pretreated rat aortic rings was tested after exposure to barium chloride ($BaCl_2$, 1 mM), 4-aminopyridine (4-AP, 1 mM), tetraethylammonium (TEA, 5 mM), and glibenclamide (10 µM). The values are expressed as mean ± SEM (n=4-5). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test. ***p<0.001 indicates a significant difference.

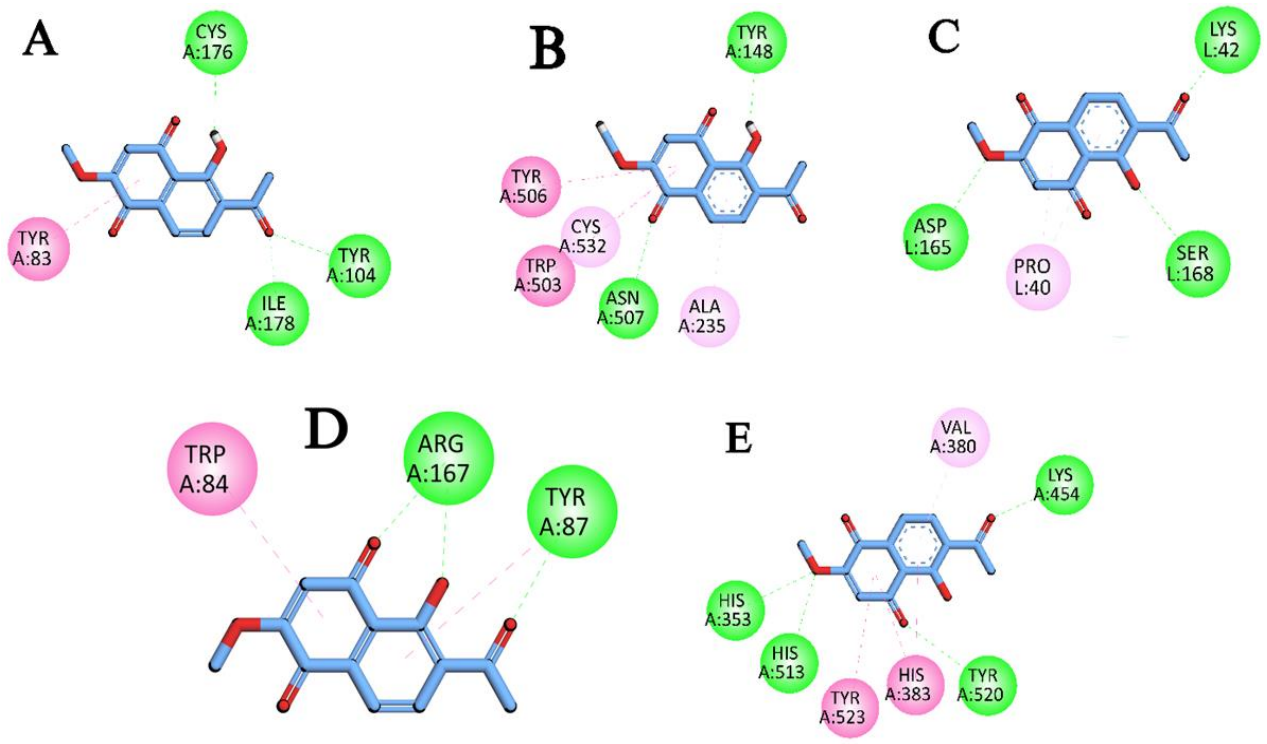

Fig. 8: 2D interaction of 2-MS with (A) muscarinic M_2 receptor (pdb id = 4MQT), (B) muscarinic M_3 receptor (pdb id = 4DAJ), (C) β_1 and β_2 receptor (pdb id = 2r4r), (D) angiotensin-II receptor (pdb id = 4ZUD) and (E) ACE enzyme (pdb id = 1O8A).

Table 1: Target proteins and their respective binding energies.

Target protein	Binding energy (kcal/mol)
4MQT (muscarinic M_2 receptor)	-6.18869066
4DAJ (muscarinic M_3 receptor)	-5.89629221
2r4r (β_1 and β_2 receptor)	-4.76055765
4ZUD (angiotensin-II receptor)	-5.07211161
1O8A (ACE enzyme)	-5.41977882

PGI_2, when it binds to the prostacyclin receptor, activates cAMP via the adenylyl cyclase (AC) pathway. Activated cAMP activates protein kinase A, which in turn reduces intracellular Ca^{2+} levels by phosphorylating calcium channels and causes vasorelaxation (White et al., 2000; Tew *et al*., 2023). To see the involvement of prostacyclin in the blood pressure lowering effect of 2-MS, normotensive rats under anesthesia were pretreated with indomethacin (10 mg/kg), a prostacyclin synthesis inhibitor (Gao and Wang, 2010). Indomethacin pretreatment did not significantly (p>0.01) reverse the fall in MAP, suggesting that prostacyclin is not a key mediator in the antihypertensive actions of 2-MS. The results indicate that 2-MS induces antihypertensive effect *in-vivo*, through a vasodilatory effect involving NO and not prostacyclin.

Blood pressure is regulated through cardiac output and peripheral vascular resistance (Tomiyama, 2023). In the blood vessels, endothelium and vascular smooth muscle cells (VSMCs) are key contributors to the regulation of blood pressure. Vascular endothelial cells synthesize and release a number of vasoactive substances that regulate vascular tone and BP. These include endothelial derived relaxing factors (EDRFs) and/or endothelial derived hyperpolarizing factors (EDHFs), endothelium-derived contracting factors (EDCFs) and endothelin (Kang, 2014). Endothelium-dependent vasorelaxation is usually dependent on EDHFs, which diffuse to the vascular smooth muscle cells and act on enzymes or receptors to cause vasorelaxation. NO and PGI_2 are well-known EDHFs in the endothelium (Kopaliani *et al*., 2024). However, Various ion channels and receptors expressed on vascular smooth muscle cells play crucial roles in regulating vascular tone. Among these, inhibition of Ca^{2+} channels and activation of K^+ channels represent key mechanisms underlying smooth muscle relaxation and vasodilation (Gollasch and Nelson, 2004).

High salt reduces endothelial nitric oxide synthase (eNOS) activity and bioavailability, thereby impairing endothelium-dependent vasodilation and promoting vascular stiffness (DuPont *et al*., 2014). We tested aortic rings from experimental rats treated with 2-MS (1, 3, and 5 mg/kg) in response to various concentrations of acetylcholine. The results show that treatment with 2-MS improved vasorelaxation in response to acetylcholine. The percent relaxation observed was 36, 53 and 80%, respectively, in comparison to the hypertensive control (16%). The data indicate that 2-MS improved response to acetylcholine probably by reversing endothelial dysfunction, improving endothelial protection and NO restoration.

Vascular function studies were performed directly on aortic rings with intact endothelium. The rings were precontracted with phenylephrine and 2-MS was added cumulatively, causing vasorelaxation with maximum relaxation observed at 100 µg/mL. This relaxation was diminished with the removal of endothelium, suggesting that endothelial mediators are involved in the vasorelaxation of 2-MS. To observe whether nitric oxide (NO) and prostanoid pathways are involved in the vasorelaxation of 2-MS, aortic rings with intact endothelium were pretreated with $_{L}$-NAME, atropine and indomethacin. Pretreatment with $_{L}$-NAME unmasked a pro-contractile component at lower concentrations that was converted to relaxation at higher concentrations. The vasoconstriction was abolished by endothelial denudation and by pretreatment with atropine and indomethacin, suggesting that the vasoconstrictive response is endothelium-dependent. The literature suggests that this paradoxical behavior is consistent with L-NAME unmasking endothelium-derived contracting factors (EDCFs), particularly COX-derived prostanoids/ thromboxane, thereby producing vasoconstriction in the absence of NO. We assume that at higher concentrations, 2-MS exerts a direct, NO-dependent vasorelaxant effect on vascular smooth muscle (VSM). The effect on VSMs, to induce vasorelaxation, are possibly due to the opening of K^+ channels or inhibition of L-type Ca^{2+} channels, thus overcoming the EDCF-mediated contraction. The same mechanisms have been previously reported for other vasoactive agents (Gluais *et al*., 2005). Preincubation with atropine reversed relaxation to 2-MS without altering the maximum response, suggesting the dominant role of the NO pathway. However, pretreatment with indomethacin did not significantly alter the vasorelaxant response induced by 2-MS, indicating that prostanoid-mediated pathways are not involved in its vasorelaxant effect. The partial attenuation of the endothelium-dependent relaxation induced by 2-MS indicates its direct action on vascular smooth muscle.

In isolated rat aortic rings, 2-MS partially relaxed (75%) high K^+ precontractions, indicating its inhibitory effect on Ca^{2+} entry. High K^+ levels trigger vascular contraction by promoting Ca^{2+} influx via voltage-gated calcium channels (VDCCs) (Li *et al*., 2024). Hence, any compound capable of inhibiting K^+ (80 mM)-induced contractions is likely to function as a calcium channel blocker (Godfraind *et al*., 1986). Thus, the inhibitory effect of 2-MS on high K^+ precontractions may predict its effects on VDCCs. Furthermore, 2-MS induced a rightward shift in $CaCl_2$-concentration response curves in Ca^{2+}-free medium, suggesting the Ca^{2+} antagonistic effect of 2-MS on voltage-operated calcium channels, similar to verapamil.

As observed earlier, 2-MS completely relaxed phenylephrine precontractions, indicating a more pronounced effect on receptor-operated calcium channels (ROCs) and/or calcium mobilization from internal stores. Phenylephrine induces vascular contraction mainly by increasing intracellular Ca^{2+} concentrations via two different pathways: Ca^{2+} release from the sarcoplasmic reticulum and its entry via ROCs. In our study, pre-incubation of aortic rings with 2-MS suppressed the formation of the phenylephrine peak in Ca^{2+} -free/EGTA medium, compared with verapamil. Our results indicate that 2-MS interferes with the mobilization of Ca^{2+} from intracellular stores, thereby contributing to its vasorelaxant action. These results partly explain the vasorelaxant mechanism of 2-MS mediated through vascular smooth muscles. It has been observed previously that high K^+ depolarizes the vascular smooth muscle membrane, leading to the closure or inactivation of potassium channels (Melaku, 2023).

To determine the role of potassium channels in the vasorelaxation induced by 2-MS, rat aortic rings were pretreated with different potassium channel blockers. The vasorelaxant effect of 2-MS was observed in the presence of barium chloride (K_{ir} channels blocker), 4-aminopyridine (K_V channels blocker), tetraethylammonium (K_{Ca} channels blocker) and glibenclamide (K_{ATP} sensitive channels blocker). The results show that only 4-aminopyridine and TEA significantly reduced the vasorelaxant response to 2-MS, suggesting activation of potassium channels. These data indicate that 2-MS possesses dual vasorelaxant effects on vascular endothelium as well as VSMCs. The vascular effects were mediated through different pathways, including NO, prostacyclin, inhibition of Ca2+ movements through VDCs, as well as release from intracellular stores and activation of potassium channels.

Data from *in-silico* molecular docking suggested binding affinity of 2-MS to various protein targets such as muscarinic M_2 and M_3 receptors, β_1 and β_2 receptors, angiotensin-II receptor and angiotensin converting enzyme (ACE). The strongest affinity was observed for muscarinic M_2 receptor (-6.1886 Kcal/mol), forming hydrogen bonds with TYR104, ILE178 and CYS176 residues and pi-pi interaction with Tyr83, Trp84 residues. These findings

highlight the muscarinic M_2 receptor as the most promising molecular target for 2-MS, accentuating its potential role in mediating the antihypertensive and vasorelaxant effects.

CONCLUSION

These data indicate that 2-MS possesses promising antihypertensive and vasorelaxant effects mediated through multiple vascular mechanisms. Chronic i.p. administration of 2-MS prevented high-salt-induced hypertension and improved vascular responsiveness. Acute intravenous administration of 2-MS produced a rapid, dose-dependent reduction in mean arterial pressure (MAP). The antihypertensive effect of 2-MS was mediated through both endothelium-dependent and -independent pathways. The endothelium-dependent component involved nitric oxide (NO) pathway, as evidenced by sensitivity to L-NAME and atropine pretreatment. In contrast, the endothelium-independent mechanism may be attributed to the inhibition of Ca^{2+} influx through voltage-operated calcium channels, suppression of Ca^{2+} release from intracellular stores and activation of K^+ channels in vascular smooth muscle. Molecular docking suggested strong bonding interactions with muscarinic M_2 receptors. All these data indicate a potent antihypertensive effect of 2-MS, mediated through multiple mechanisms and may act as a promising natural antihypertensive agent through synergistic endothelial and smooth muscle mechanisms.

Acknowledgments
The authors gratefully acknowledge the financial support of the Higher Education Commission (HEC), Pakistan, under the Indigenous (5000) Scholarship Program, which significantly contributed to the successful completion of this research. The authors are also thankful to the Department of Pharmacy, COMSATS University Islamabad, Abbottabad Campus, for the lab facilities.

Authors' contribution
Adil Javed and Abdul Jabbar Shah: Conceptualization; Adil Javed, Taseer Ahmed, Muhammad Ikram and Abdul Jabbar Shah: Methodology; Adil Javed: Validation, writing—original draft; Adil Javed, Taseer Ahmed and Abdul Jabbar Shah: Project administration and visualization; Taseer Ahmed, Muhammad Ikram and Abdul Jabbar Shah: Supervision; Muhammad Ikram and Abdul Jabbar Shah: writing—review and editing; Atif Ali Khan Khalil and Mi-Jeong Ahn: Isolated 2-Methoxystypandrone. All authors have read and agreed to the published version of the manuscript. We declare that the current work was carried out by the authors listed in this article.

Funding
The author(s) gratefully acknowledge the financial support of the Higher Education Commission (HEC), Pakistan, under the Indigenous (5000) Scholarship Program, which significantly contributed to the successful completion of this research.

Data availability statement
All data generated or analysed during this study are included in this published article [and its supplementary information files].

Ethical approval
The experiments were conducted on Sprague-Dawley rats, approved by the Research Ethics Committee, Department of Pharmacy, CUI, Abbottabad Campus (PHM.Eth/CS-M02-07-2975) and conformed with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 2010. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest
The authors declare no conflict of interest.

Supplementary data

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