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Original Research Article

Antihypertensive and Vasorelaxant Effects of *Cistanche phelypaea* Leaves through Inhibition of Receptor-Operated Calcium Channels in Rats

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ABSTRACT

Cistanche phelypaea (family: Orobanchaceae) is a medicinal plant traditionally used for its vasorelaxant, diuretic, and sedative properties, highlighting its potential relevance in the management of hypertension. The present study aimed to evaluate the antihypertensive and vasorelaxant effects of *Cistanche phelypaea* leaves and its impact on angiotensin-converting enzyme 2 (ACE-2). The aqueous leaf extract of *Cistanche phelypaea* (CPAE) was prepared and its antihypertensive effect (200 mg/kg) was examined using a hypertensive rat model induced by oral administration of L-NAME (60 mg/kg bw), additionally, we assessed its vasorelaxant potential and its impact on the stimulation or inhibition of ACE-2 in isolated rat thoracic aorta. The results indicate that CPAE significantly lowered systolic ($p<0.0001$), diastolic ($p<0.01$), and mean arterial pressure ($p<0.01$) without affecting normotensive rats. The data showed that CPAE mediates its antihypertensive effect via vasodilatory activity. Furthermore, the vasorelaxant capacity of CPAE seems to be mediated through receptor-operated calcium channels (ROCCs). However, CPAE had no effect on ACE-2. In conclusion, the study demonstrates that the aqueous leaf extract of *Cistanche phelypaea* possesses a significant antihypertensive and vasorelaxant effects, primarily through the inhibition of Ca^{2+} entry.

Keywords: *Cistanche phelypaea*, Medicinal plant, L-NAME, Blood pressure, Hypertension, Aortic ring, Vasorelaxant.

Introduction

Hypertension, an important worldwide public health challenge, is a leading cause of cardiovascular, retinal and kidney diseases.[1] The management of hypertension is based on the use of antihypertensive drugs such as diuretics, *β*-blockers, calcium channels blockers, angiotensin II receptor blockers (ARBs), angiotensin-converting enzyme (ACE) inhibitors, direct vasodilators, and so on. However, the multitude of side effects associated with these medications, including muscle cramps, extreme tiredness, dizziness, blurred vision, dehydration, skin rash, edema, renal dysfunction, and others, constitutes a veritable problem.[2] Consequently, an increasing number of patients are opting for traditional natural products as an alternative approach to managing hypertension.[3]According to ethnopharmacological surveys conducted in three regions of Morocco, a significant percentage (67.5% to 80%) of patients rely on medicinal plants for the treatment of hypertension.[4-6] The Cistanche genus, which belongs to the Orobanchaceae family, comprises 22 species of perennial parasitic plants mainly distributed in arid and semi-arid zones and in the deserts of the northern hemisphere.[7] Cistanche species are highly regarded as potent therapeutic agents and are commonly known as the "Ginseng of the deserts". They are used for the treatment of various health issues, including chronic renal disease, impotence, female infertility, morbid leukorrhea, and profuse metrorrhagia.[8] Furthermore, they have been demonstrated to possess sedative, vasorelaxant, and aphrodisiac effects and to enhance cognitive abilities and improve learning skills.[9] *Cistanche phelypaea* (L.) Cout is an edible holoparasitic plant that grows naturally in sand dunes in Portugal, Spain, Crete, Cyprus, Turkey and the eastern Mediterranean region.[9] The thick lower part of its stem has traditionally been employed to treat abdominal pain, diarrhea, muscle contractions, and ecchymosis[10] and has also been used as diuretic.[11] In addition, the underground part of the plant is used for treating intestinal disorders and diabetes.[12] In Morocco, locals in the Tissint region use the powdered aerial parts of the plant as a haemostatic agent.[10,11] However, to date, no pharmacological studies have been carried out to assess the antihypertensive effect of *Cistanche phelypaea*. Therefore, the present study was conducted to evaluate, for the first time, the antihypertensive effect of the aqueous extract of *Cistanche phelypaea* leaves in N ω - Nitro-L-arginine methyl ester (L-NAME)-induced hypertensive and normotensive rats, and to determine the specific signaling pathways involved in its vasorelaxant activity in addition to its impact on angiotensin-converting enzyme-2 (ACE-2).

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Materials and Methods

Chemical reagents and drugs

Epinephrine (EP), acetylcholine, N(ω)-nitro-L-arginine methyl ester (L-NAME), methylene blue (MB), MLN-4760, diminazene aceturate (DIZE), indomethacin, barium chloride ($BaCl_2$) and 4-aminopyridine (4-AP) were purchased from Sigma Chemical Co. (St. Louis, USA). atropine was purchased from ChemCruz. All additional reagents were of analytical grade and from local sources. All drugs mentioned were dissolved in distilled water.

Plant material and extraction

The leaves of *Cistanche phelypaea* were collected from Boudnib (southeast of Morocco) in April 2023. The plant was taxonomically identified and authenticated by the Department of Botany, Faculty of Sciences and Techniques Errachidia, and a voucher specimen (N° CP03) was deposited at our faculty's herbarium. 1 g of leaf powder was mixed with 100 ml of distilled water and boiled for 10 minutes. The mixture was then allowed to cool to room temperature for 15 minutes. Thereafter, the aqueous extract was filtered to remove particulate matter. The filtrate obtained was lyophilized.[13]

Experimental animals

Healthy adult male albino Wistar rats, weighing between 150 and 250 g, were obtained from the Experimental Center of Missour (Morocco). The animals were housed individually in polyethylene cages under standard laboratory conditions (23 ± 1°C, 55 ± 5% humidity, and a 12 h/12 h light/dark cycle) and maintained with free access to drinking water and were fed a standard laboratory pellet diet ad libitum All experiments were conducted according to the guidelines of the local committee of the Faculty of Sciences & Techniques Errachidia, Morocco, with ethical approval number: FSTE/2015.

Induction of hypertension

Arterial hypertension was induced in male Wistar albino rats by oral administration of L-NAME at a dose of 60 mg/kg body weight once daily for seven consecutive days. The animals that were confirmed to be hypertensive, with systolic blood pressure ≥ 150 mmHg, were included in the study.[14]

Blood pressure measurements

Normotensive and hypertensive male rats were randomly divided into four groups of six animals each. The first, a control group, received distilled water, the second group was treated with an aqueous extract of *Cistanche phelypaea* leaves at a dose of 200 mg/kg of body weight, while the third group received furosemide at a dose of 20 mg/kg, used as a reference drug. Treatments were administered orally, either as a single dose for the acute assessment or daily for seven consecutive days for the subacute study. For the single oral administration (acute test), animals received either distilled water (control group), furosemide or CPAE, the systolic blood pressure (SBP), mean arterial blood pressure (MBP), and heart rate (HR) were measured at baseline (t_0) and six hours post-administration (t_6) to assess short-term effects. For the repeated oral administration, treatments were given once daily for 7 consecutive days, and the SBP, MBP, and HR were monitored throughout this sub-acute period. To ensure reproducible blood pressure measurements, the animals were warmed (approximately 35°C) for 15 to 20 minutes, then anesthetized to avoid the stress of immobilization. Subsequently, they were placed in non-invasive blood pressure (NIBP) restraints, and a cuff equipped with a sensor was positioned on their tails for data acquisition. Three consecutive measurements were obtained per animal using the tail cuff method coupled with a computer-assisted monitoring system. The cuff was inflated to a pressure greater than 200 mmHg and then gradually deflated, during which pulse rates were recorded using the PowerLab data acquisition system and LabChart version 5.0 software (Harvard Apparatus, Boyer, Casablanca, Morocco). The SBP, MBP, and HR were directly derived from pulse tracings, while the DBP was calculated using the formula: DBP = (3MBP - SBP)/2. All blood pressure measurements were conducted at the same time each day throughout the experimental period (7 days).[14]

Measurement of vascular relaxation, evaluation of mechanisms involved

Male Wistar rats (260–300 g) were sacrificed by stunning and exsanguination after anesthesia with pentobarbital sodium (50 mg/kg). The aortas were immediately excised and placed in a cold buffer and were then quickly removed and carefully cleaned of adhering fat and connective tissue. The isolated arteries were transversely cut into rings measuring 2 to 3 mm in length and mounted under a resting tension of 2 g in a tissue bath containing 40 mL of Krebs-Henseleit (KH) solution continuously oxygenated (95% O_2, 5% CO_2) and maintained at 37 °C and a pH of 7.4. The aortic rings were mounted between two stainless steel hooks, one attached to the base of the chamber and the other connected to a UF1 force transducer (LCM Systems Ltd) to record isometric tension. All experiments were conducted on endothelial-intact aortic rings. The functional integrity of the endothelium was verified by the ability of acetylcholine (10^{-5} M) to induce at least 40–60% relaxation in aortic rings precontracted by epinephrine (10^{-5} M). Thoracic aortic rings were subjected to an optimal initial tension of 2 g (100%), determined from preliminary experiments to be the tension that induces the maximal contractile response to epinephrine (10^{-5} M). After an equilibration period of 1 hour, the rings were contracted by the addition of 10 µM epinephrine (EP) or 80 mM potassium chloride (KCl) to assess their contractile reactivity after exposure to EP or KCl. To restore basal tension, aortic rings were washed three times with Krebs solution which was renewed every 60 minutes to maintain the physiological conditions of the tissues in the bath. To establish cumulative dose-response curves of CPAE-induced vasorelaxation, rat aortic rings were first equilibrated and then contracted by EP (10 µM) or KCl (80 mM). Once the contraction plateau was reached, CPAE (0.5, 1, 1.5 and 2 mg/ml) was added cumulatively at 10-minute intervals. To explore the mechanisms underlying the observed vasorelaxant effect, a series of experiments were performed on aortic rings pre-contracted by EP. Thus, the following experiment was conducted: Before adding EP, the aortic rings were pre-incubated for 20 min with one of the following standard drugs: 1) 10^{-4} M L-NAME, a direct inhibitor of nitric oxide (NO) Synthase. 2) 10^{-5} M indomethacin, a prostaglandin synthesis inhibitor.3) 10^{-5} M nifedipine, a L-type calcium channel blocker. 4) 10^{-5} M methylene blue (MB), a Guanylate cyclase inhibitor. 5) 10^{-5} M propranolol, a beta-blocker. 6) 10^{-5} M atropine a muscarinic receptor antagonist. 7) 10^{-5} M glibenclamide, an ATP-sensitive K+ channel blocker. 8) 10^{-5} M barium chloride ($BaCl_2$) an inhibitor of inwardly rectifying potassium channels (a K_{IR} blocker). 9) 10^{-4} M 4-aminopyridine (4-AP) a voltage dependent K+ channel inhibitor (a KV blocker), and 10) 6 nM MLN-4760 an ACE-2 inhibitor. Following the addition of EP (10^{-5} M), the relaxation of aortic rings was achieved by the cumulative addition of CPAE (0.5, 1, 1.5, and 2 mg/ml). The vasorelaxant effect on aortic rings was calculated and expressed as a percentage of maximal contraction in response to EP. Between each treatment, aortic rings were washed thoroughly three times, with five-minute incubation intervals for each wash, to ensure the full clearance of previously applied substances. The control was conducted by the cumulative addition of distilled water to aortic rings precontracted by EP (10 µM) and KCl (80 mM).[15]

Effects of CPAE on Extracellular Calcium-induced Contraction

In order to determine the role of calcium channels in the vasodilator activity of CPAE, the following experimental procedure was carried out on the basis of previous studies, with some slight modifications,[14] the effect of CPAE (0.5 mg/ml) was tested after EP or KCl pre-treatment in a buffer containing $CaCl_2$ or Ca^{2+}-free. The contraction response induced by $CaCl_2$ (0.5, 1, 3 mM) in the aortic rings pre-treated by EP (10^{-5} M) or KCl (80 mM) was evaluated as well as in Ca^{2+}-free KH buffer. The contraction responses induced by $CaCl_2$ were expressed in grams (g) in the presence and absence of CPAE pretreatment.

ACE-2 Inhibition Test

To establish cumulative dose-dependent curves for Diminazene-induced relaxation, aortic rings were precontracted with epinephrine (10^{-5}M). Once the plateau phase was achieved, diminazene aceturate (10^{-4}M) was added. The control was established by cumulatively adding a vehicle (distilled water) to aortic rings pre-contracted by EP (10^{-5}M). In order to investigate the potential inhibition of ACE2 by CPAE, the following experiment was carried out as it has been previously described with some slight modifications[16]: aortic rings were pre-incubated with CPAE (0.375 mg/ml) for 20 min prior to EP addition. After the plateau was reached, diminazene aceturate (10^{-4}) was added. The inhibitory effect of CPAE was assessed as its ability to inhibit the vasorelaxation induced by diminazene.

Statistical analysis

All data were expressed as mean ± SEM. Statistical significance between multiple groups was tested using two-way ANOVA followed by the Bonferroni for multiple comparisons test. Relaxations induced

by the aqueous extract of *Cistanche phelypaea* leaves were expressed as a percentage decrease of epinephrine (EP) and KCl contraction. Each cumulative dose-effect curve for *Cistanche phelypaea* aqueous extract (CPAE) induced relaxation was plotted, contingent on application of the sigmoidal curve fitting and non-linear regression. Statistical analyses were performed using Prism version 8 (GraphPad Software Inc., San Diego, CA., U.S.A.) to generate Rmax (maximal relaxant response) and EC_{50} (the concentration of CPAE dose required to give 50% of the maximum aortic-relaxant response). Differences were considered to be statistically significant when $p<0.05$.

Results and Discussion

Anti-hypertensive effect of CPAE

Following the daily administration of L-NAME a marked elevation in systolic blood pressure levels was observed, confirming the successful induction of hypertension. Table **1** presents the effects of the aqueous extract of *Cistanche phelypaea* (CPAE) on blood pressure levels in both normal and L-NAME hypertensive rats after a single oral administration (after 6 hours of CPAE treatment) of the extract at a dose of 200 mg/kg. No significant change in arterial blood pressure levels (systolic, mean and diastolic blood pressure) or heart rate was observed in either normotensive or hypertensive rats after the single oral administration of CPAE. Regarding furosemide, blood pressure levels (systolic, mean and diastolic) and heart rate were unchanged in normotensive rat groups after six hours of oral administration of the reference drug.

Table 1: Effect of a single oral administration of CPAE (200 mg/kg) on SBP, MBP, DBP (mm Hg), and heart rate (bmp) in normal and L-NAME-induced hypertensive rats

Groups		Systolic blood pressure (mm Hg)		
		Control	**CPAE**	**Furosemide**
Normal	**t0**	132.00 ± 6.00	129.45 ± 3.64	120.00 ± 7.00
	t6	138.00 ± 8.00	130.37 ± 3.53	111.00 ± 7.00
L-NAME	**t0**	180.00 ± 5.00	193.87 ± 4.56	169.00 ± 7.00
	t6	175.00 ± 7.00	187.05 ± 3.45	142.00 ± 6.00*
		Mean blood pressure (mm Hg)		
		Control	**CPAE**	**Furosemide**
Normal	**t0**	115.00 ± 7.00	105.12 ± 3.58	108.00 ± 6.00
	t6	113.00 ± 6.00	100.37 ± 4.28	97.00 ± 2.00
L-NAME	**t0**	156.00 ± 6.00	150.77 ± 2.49	150.00 ± 3.00
	t6	157.00 ± 6.00	137.17 ± 2.33	125.00 ± 6.00**
		Diastolic blood pressure (mm Hg)		
		Control	**CPAE**	**Furosemide**
Normal	**t0**	106.00 ± 5.00	92.95 ±1.55	103.00 ± 5.00
	t6	101.00 ± 6.00	85.37 ± 4.40	91.00 ± 4.00
L-NAME	**t0**	144.00 ± 7.00	129.22 ± 4.95	152.00 ± 5.00
	t6	148.00 ±10.00	112.23 ±3.29	129.00 ± 4.00*
		Heart rate (bpm)		
		Control	**CPAE**	**Furosemide**
Normal	**t0**	320.00 ±10.00	285.45 ± 26.24	349.00 ± 19.00
	t6	306.00 ± 8.00	276.94 ± 25.94	321.00 ± 9.00
L-NAME	**t0**	312.00 ± 15.00	300.46 ±19.32	348.00 ± 22.00
	t6	318.00 ± 12.00	298.65 ± 13.92	311.00 ± 17.00

Data are expressed as means ± SEM, n=6. *p<0.05, **p<0.01, ***p<0.001.

However, in hypertensive rats treated with furosemide, we observed a considerable decrease in mean ($p<0.01$), systolic and diastolic ($p<0.05$) blood pressure values when compared to the baseline (t0). Additionally, Table **2** shows the evolution of systolic, mean and diastolic blood pressure values and heart rate in normotensive and hypertensive rats over 7 days of repeated oral administration of CPAE (200 mg/kg). The results showed that the repeated oral administration of CPAE did not induce any significant changes in blood pressure levels or heart rate in normotensive rats during the treatment period. However, in hypertensive rats, the results revealed that treatment with CPAE significantly reduced systolic blood pressure (SBP) on the 2nd, 4th, and 7th days of treatment ($p<0.0001$). Additionally, the treatment of hypertensive rats with furosemide significantly reduced systolic blood pressure on the 2nd, 4th, and 7th days ($p<0.0001$). Similarly, the treatment of normotensive group with the reference drug significantly reduced SBP ($p<0.01$).

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Table 2: Effect of repeated oral administration of CPAE (200 mg/kg) on SBP, MBP, DBP (mmHg), and heart rate (bmp) in normal and L-NAME-induced hypertensive rats.

Groups		Systolic blood pressure (mm Hg)		
		Control	**CPAE**	**Furosemide**
Normal	**D0**	134.00 ± 7.00	129.45 ± 3.64	120.00 ± 7.00
	D2	136.00 ± 5.00	128.16 ± 3.54	115.00 ± 5.00
	D4	140.00 ± 3.00	125.16 ± 5.45	108.00 ± 3.00
	D7	135.00 ± 6.00	124.14 ± 4.41	99.00 ± 4.00**
L-NAME	**D0**	180.00 ± 5.00	193.87 ± 4.56	169.00 ± 6.00
	D2	176.00 ± 5.00	154.80 ± 3.58****	122.00 ± 2.00****
	D4	173.00 ± 9.00	143.50 ± 3.94****	120.00 ± 4.00****
	D7	172.00 ± 6.00	145.66 ± 3.76****	109.00 ± 3.00****
		Mean blood pressure (mm Hg)		
		Control	**CPAE**	**Furosemide**
Normal	**D0**	111.00 ± 4.00	105.12 ± 3.58	109.00 ± 7.00
	D2	112.00 ± 5.00	102.34 ± 4.74	107.00 ± 5.00
	D4	117.00 ± 6.00	100.00 ± 3.42	101.00 ± 3.00
	D7	112.00 ± 4.00	99.83 ± 4.44	96.00 ± 2.00
L-NAME	**D0**	156.00 ± 6.00	150.77 ±3.49	150.00 ± 3.00
	D2	149.00 ± 6.00	126.50 ± 3.09**	148.00 ± 5.00
	D4	148.00 ± 9.00	120.50 ± 4.64***	103.00 ± 5.00****
	D7	147.00 ± 6.00	118.60 ± 3.51***	95.00 ± 4.00****
		Diastolic blood pressure (mm Hg)		
		Control	**CPAE**	**Furosemide**
Normal	**D0**	100.00 ± 3.00	92.95 ±3.55	103.00 ± 6.00
	D2	101.00 ± 5.00	89.43 ± 5.34	98.00 ± 5.00
	D4	106.00 ± 3.00	87.42 ± 2.40	97.00 ± 6.00
	D7	102.00 ± 8.00	87.67 ± 3.34	95.00 ± 4.00
L-NAME	**D0**	144.00 ± 7.00	129.22 ±2.95	152.00 ± 5.00
	D2	135.00 ± 7.00	112.35 ± 2.84*	103.00 ± 4.00****
	D4	135.00 ± 6.00	109.00 ± 5.00*	100.00 ± 4.00****
	D7	136.00 ± 6.00	105.07 ± 3.38**	98.00 ± 3.00****
		Heart rate (bpm)		
		Control	**CPAE**	**Furosemide**
Normal	**D0**	312.00 ± 15.00	285.45 ± 26.24	348.00 ± 22.00
	D2	320.00 ± 14.00	272.20 ± 24.22	341.00 ± 16.00
	D4	312.00 ± 16.00	280.53 ± 16.87	341.00 ± 17.00
	D7	319.00 ± 8.00	276.73 ± 14.97	305.00 ± 8.00
L-NAME	**D0**	308.00 ± 12.00	300.46 ± 19.32	348.00 ± 15.00
	D2	310.00 ± 15.00	294.86 ± 3.75	343.00 ± 13.00
	D4	307.00 ± 13.00	297.73 ± 2.71	315.00 ± 11.00
	D7	318.00 ± 18.00	289.13 ± 8.97	311.00 ± 10.00

Data are expressed as means ± SEM, n=6. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Likewise, the treatment of hypertensive rats with CPAE resulted in a significant decrease of MBP (mean arterial pressure) on the 2nd ($p<0.01$), 4th, and 7th ($p<0.001$) days of treatment. Furosemide treatment resulted in a significant decrease of mean arterial blood pressure on the 4th and 7th days ($p<0.001$) in hypertensive rats; however, no noticeable effect was observed in the normotensive groups receiving furosemide. Similarly, the repeated oral administration of CPAE (200 mg/kg) of hypertensive rats significantly decreased the diastolic blood pressure levels at the second, fourth ($p<0.5$), and seventh day of treatment ($p<0.01$). Additionally, furosemide significantly reduced DBP on the 2nd, 4th, and 7th days ($p<0.0001$) in hypertensive rats, while it showed no impact on DBP in normotensive rats. Our results are consistent with those of a previous study, which demonstrated that polysaccharides from *Cistanche deserticola* induced a significant reduction in blood pressure values (SBP and DBP) in hypertensive rats [17]. On other hand, repeated oral treatment with CPAE had no effect on heart rate in either normotensive or hypertensive rats, suggesting that the observed antihypertensive effect of CPAE is likely due to modulation of vascular mechanisms. Indeed, it is important to note that one of the main mechanisms of action of antihypertensive drugs is to reduce vascular resistance by inducing direct or indirect dilation of blood vessels.[18] Therefore, in order to determine one of the mechanisms of action involved in the antihypertensive effect of *Cistanche phelypaea* aqueous extract, an *in vitro* study was carried out to assess its vasorelaxant activity on aortic rings isolated from Wistar rats.

Measurement of vascular relaxation and evaluation of mechanisms involved

The vasorelaxant effect of CPAE on pre-contracted aortic rings by both EP and KCl

According to the results of the present study, cumulative concentrations of CPAE (0.5, 1, 1.5 and 2 mg/ml) induced a vasodilatory effect on aortic rings precontracted by EP (Figure 1A), observable from the second concentration (1 to 2 mg/ml, $p<0.0001$) (Rmax = 71.90±5.58 %) while the cumulative addition of CPAE did not demonstrate any significant vasodilator effect in aortic rings pre-contracted with KCl (80mM) (Figure 1B), Suggesting that CPAE exerts its vasorelaxant activity by specifically targeting ROCCs (receptor-operated calcium channels) and not VDCCs (voltage-dependent calcium channels).

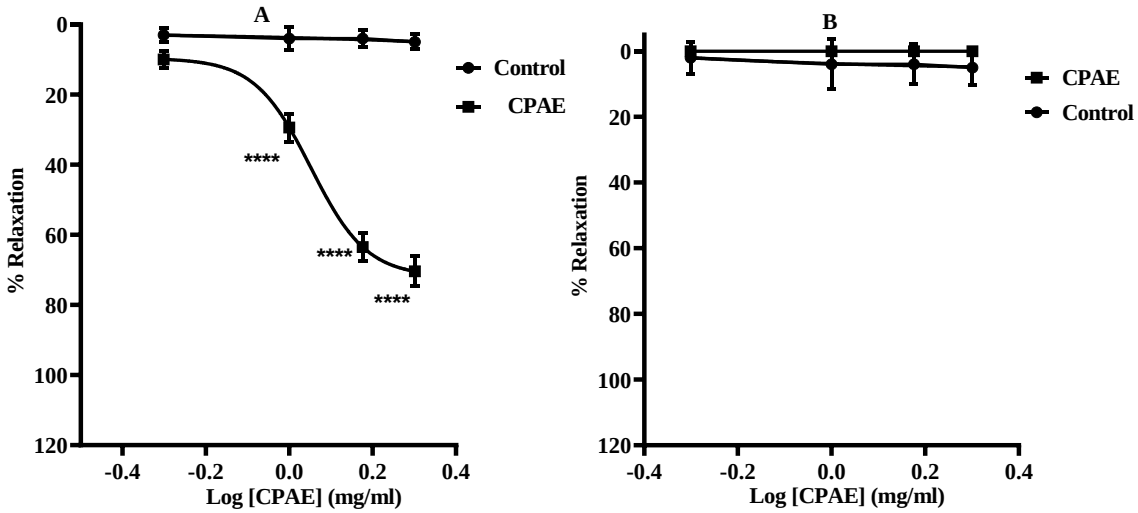

Figure 1: Vasorelaxant effect of CPAE (0.5, 1, 1.5 and 2 mg/ml) on aortic rings precontracted with EP (10µM) (**A**) or KCl (80 mM) (**B**). Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs control.

Aortic responses to CPAE in the absence and presence of L-NAME or indomethacin

The results showed that pre-incubation of the aortic rings with L-NAME (10^{-4} M) did not significantly inhibit CPAE-induced relaxation (Rmax=81.53±4.28 %) (Figure 2A). Similarly, indomethacin did not significantly reduce aortic ring dilation in response to CPAE doses (Rmax=80.07±8.97 %) (Figure 2B). These results indicate that the vasorelaxant effect of CPAE is not mediated through nitric oxide or prostaglandin signaling pathways.

Involvement of NO-cyclic guanosine monophosphate (cGMP) and calcium channel blocker pathway

The findings indicated that pre-incubation of the aortic ring with nifedipine or methylene blue did not inhibit CPAE-induced vasorelaxation. Interestingly, the appropriate values obtained in aortic rings treated only with CPAE were: Rmax=71.90±5.58 %. In contrast, the values obtained in aortic rings pre-incubated with nifedipine were: Rmax=75.82±6.49 % (Figure 3A), whereas for rings treated with MB, the values were: Rmax=94.50±15.78 % (Figure 3B). These findings suggest that the observed vasorelaxant effect of CPAE is not mediated through the L-type calcium channels pathway or the soluble guanylate cyclase (sGC)/cGMP signaling pathway.

Role of muscarinic and ß-adrenergic receptors in CPAE-induced vasorelaxation

The results showed that CPAE-induced vasorelaxation was unaffected by atropine pre-treatment (CPAE: Rmax=71.90±5.58 % vs CPAE+Atropine: Rmax=104.6±29.59 %) (Figure 4A). Likewise, incubation of aortic rings with propranolol did not significantly reduce the vasorelaxant effect of CPAE (CPAE: Rmax=71.90±5.58% vs CPAE+Propranolol: Rmax=112.3±33.35 %) (Figure 4B), suggesting that the CPAE-induced vasorelaxation does not appear to be mediated by muscarinic or β-adrenergic pathways.

Vasorelaxant effect of CPAE in the presence of potassium channels blockers, Glibenclamide, $BaCl_2$, or 4-Aminopyridine

As illustrated in Figure 5, the results showed that the vasorelaxant effect of CPAE was not affected by the pre-treatment of aortic rings with glibenclamide (an ATP-sensitive potassium channels blocker), $BaCl_2$ (an inwardly rectifying potassium channels inhibitor, and 4-aminopyridine (voltage dependent K^+ channels inhibitor), with EC_{50} and Rmax values of 1.359±0.113 mg/ml and 104±34.67% for glibenclamide, 1.46±0.027 mg/ml and 74.54±5.04 % for $BaCl_2$ and 1.512±0.053 mg/ml and 102.5±20.78 % for 4-aminopyridine, respectively, which discarded the possible intervention of potassium channels in the vasorelaxant effect of CPAE.

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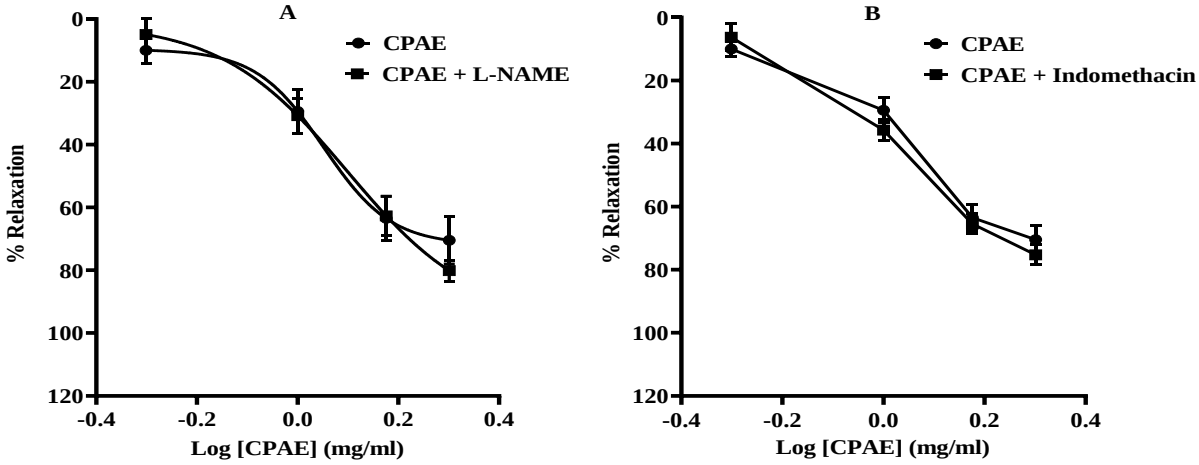

Figure 2: Concentration-response curves for the vasorelaxant effect of CPAE on EP-precontracted aortic rings, in the presence of L-NAME **(A)** and Indomethacin **(B)**. Data represent mean ± SEM.

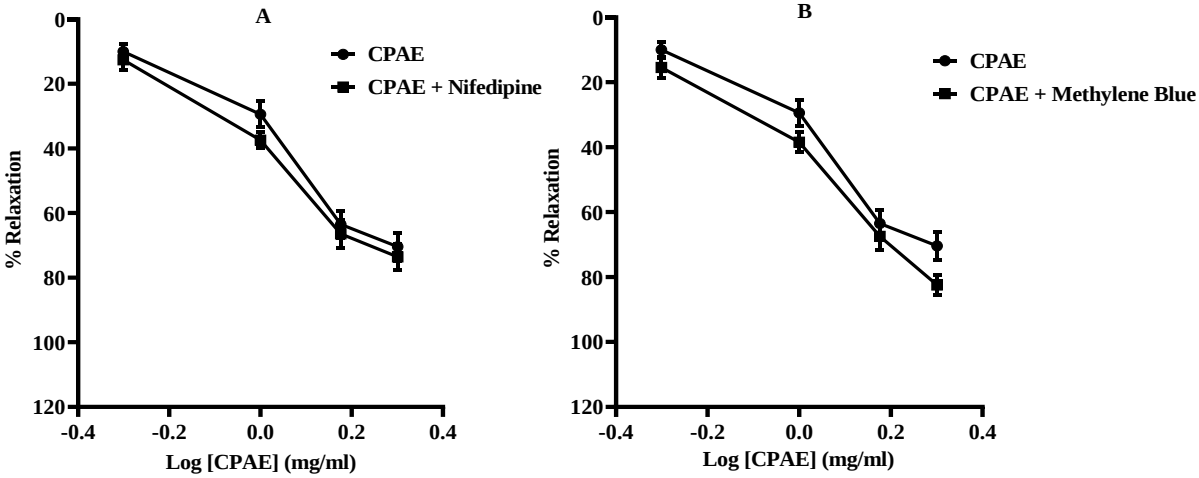

Figure 3: Concentration-response curves for the vasorelaxant effect of CPAE on EP-precontracted aortic rings, in the presence of Nifedipine **(A)** and Methylene blue **(B)**. Data represent mean ± SEM.

Effect of CPAE on extracellular Ca^{2+}-induced contraction

In a Ca^{2+}-free KH buffer, the cumulative addition of $CaCl_2$ (0.5, 1, 3 mM) induced an increase of tension in the rat thoracic aorta rings, reflecting the activation of membrane calcium channels. As illustrated in Figure 6, pretreatment with CPAE (0.5 mg/ml) for 20 min significantly ($p<0.001$) inhibited the prolonged contractions induced by the addition of cumulative concentrations of extracellular $CaCl_2$ (0.5, 1, 3 mM) in aortic rings pre-contracted by EP (Figure 6A). However, the same pre-treatment with CPAE did not exhibit any inhibitory effect on the prolonged contractions induced by extracellular $CaCl_2$ compared to the control (Figure 6B) in aortic rings precontracted by KCL. It is known that entry of extracellular Ca^{2+} through voltage-dependent calcium channels (VDCCs) or receptor-operated calcium channels (ROCCs) and the intracellular release of Ca^{2+} by the sarcoplasmic reticulum led to an increase in intracellular calcium levels, thus triggering vascular smooth muscle contraction.[19] Consequently, the relaxation of vascular smooth muscle can be achieved by inhibiting the Ca^{2+} influx required for excitation-contraction coupling. Epinephrine is an alpha-adrenergic agonist that induces the contraction of vascular muscle cells by an influx of Ca^{2+} through the ROCCs and by the release of intracellular Ca^{2+} from the sarcoplasmic reticulum, triggered by the activation of IP3 receptors.[20,21] In contrast, KCl contracts smooth muscle cells mainly through the influx of extracellular Ca^{2+} resulting from the depolarization of the cell membrane and the subsequent opening of the VDCCs.[22]In the light of these results, it can therefore be concluded that the vasorelaxant effect of CPAE appears to be mediated by selective inhibition of extracellular Ca^{2+} influx via receptor-operated calcium channels (ROCCs), without affecting voltage-dependent calcium channels (VDCCs). These results are comparable to those obtained previously by Yoshikawa et al,[23] who showed in their study that certain active constituents of the ethanolic extract from the dried stems of *Cistanche tubulosa* inhibited contractions via receptor-operated calcium channels, but not via voltage-dependent calcium channels. Likewise, our findings are similar to those reported by Owolabi et al., who demonstrated that the vasorelaxant effect of the aqueous extract of *Hibiscus sabdariffa* petals was mediated through a selective inhibition of extracellular calcium mobilization via receptor-operated calcium channels.[24]

Effect of CPAE on vascular angiotensin-converting enzyme 2 (ACE-2) Stimulating ACE-2

The outcomes of the current study revealed that the pre-incubation of aortic rings with MLN-4760 (1 nM) did not modify the relaxant effect of aortic rings in response to various CPAE doses. Indeed, in vehicle-treated rings, the corresponding values were: EC_{50}=1.126±0.028 mg/ml and Rmax=71.90±5.58 %, whereas in aortic rings pre-incubated with MLN-4760, the values were: EC_{50}=1.131±0.02 mg/ml and Rmax=82.37±5.15 % (Figure 7).

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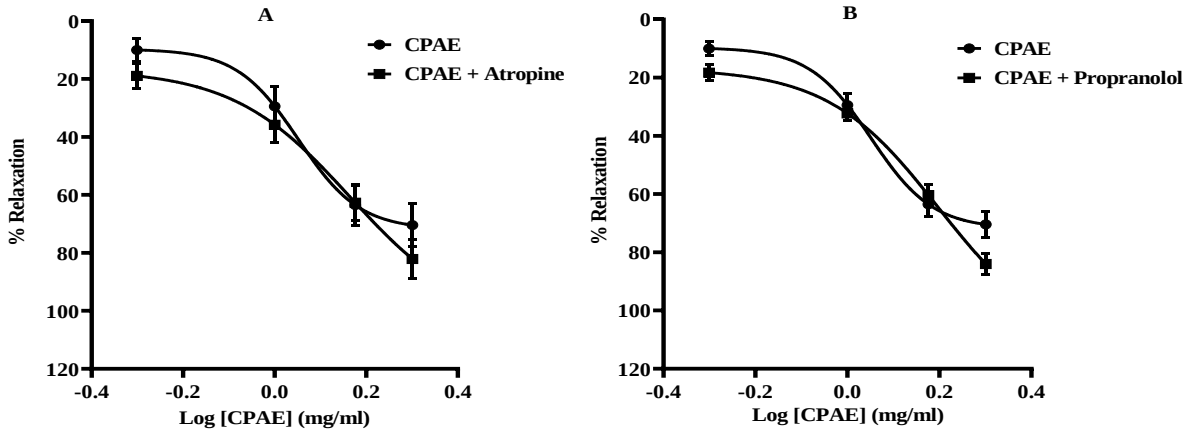

Figure 4: Concentration-response curves for the vasorelaxant effect of CPAE on EP-precontracted aortic rings, in the presence of Atropine **(A)** and Propranolol **(B)**. Data represent mean ± SEM.

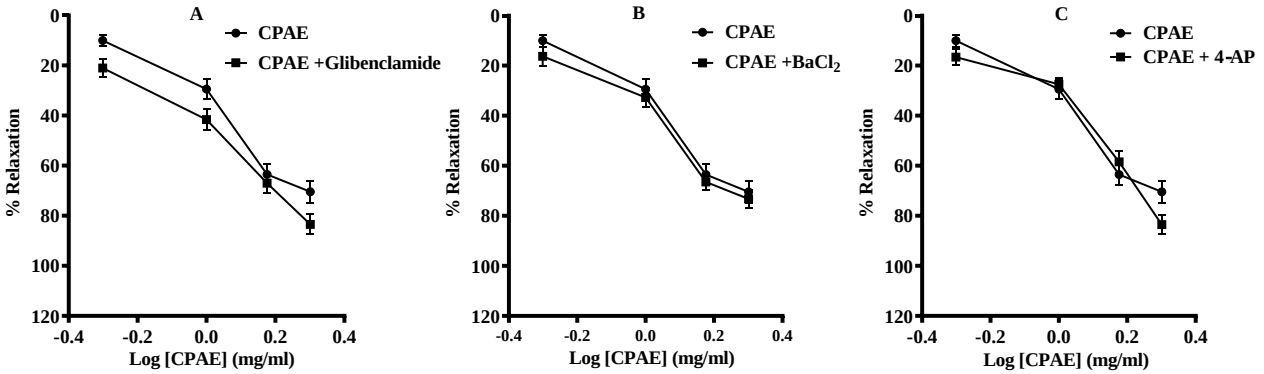

Figure 5: Concentration-response curves for the vasorelaxant effect of CPAE on EP-precontracted aortic rings, in the presence of the following Potassium channel inhibitors: **(A)** Glibenclamide, **(B)** $BaCl_2$, **(C)** 4-Aminopyridine (4-AP). Data represent mean ± SEM.

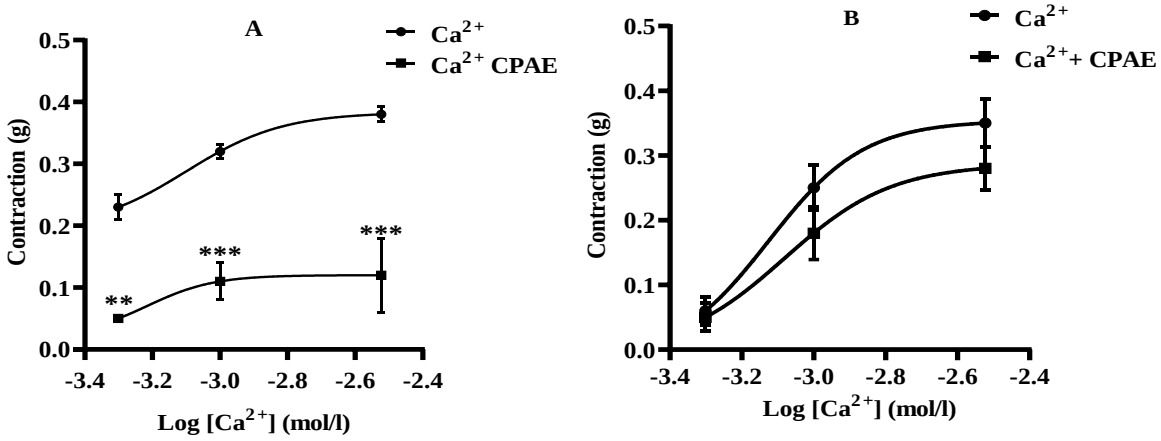

Figure 6: Inhibitory effect of CPAE (0.5 mg/ml) on the contraction induced by extracellular Ca^{2+} in aortic rings pre-contracted with EP (10 μM) (A) or KCl (80 mM) (B) in the presence (squares) or absence (circles) of CPAE. Values are expressed as mean ± SEM. **p<0.01, *** p<0.001, **** p<0.0001 compared with control.

Widely recognized as a critical regulator of the renin-angiotensin system (RAS), angiotensin-converting enzyme 2 (ACE-2), is expressed in multiple tissues, including the lungs, heart, kidneys, vascular smooth muscle cells, gastrointestinal tract, and brain, suggesting its ubiquitous distribution, it acts by cleaving angiotensin I (Ang I) to generate Ang 1-9 and degrading Ang II to Ang 1-7, a vasodilatory peptide that acts via Mas receptors to induce vascular relaxation.[25] In the present study, MLN-4760, a specific ACE-2 inhibitor, was used to determine whether the vasorelaxant action of

CPAE is mediated by the ACE-2 stimulation pathway. Our results revealed that the pretreatment with MLN-4760 had no effect on the vasodilatory effect of CPAE, suggesting that CPAE-induced vasorelaxation is independent of ACE2 activation.

Inhibiting ACE-2

The addition of diminazene (10^{-4} M) to aortic rings precontracted by EP resulted in a significant relaxation ($p<0.0001$). To determine whether the aqueous extract of *Cistanche phelypaea* (CPAE) exerts an inhibitory effect on ACE-2, aortic rings were preincubated with CPAE (0.375 mg/ml) for 20 minutes before being precontracted with EP. The results illustrated in Figure 8 showed that pre-treatment with CPAE did not affect DIZE-induced vasorelaxation in aortic rings precontracted by EP (Rmax=65.70±5.21 % in the presence of CPAE vs Rmax=54.36±3.5 9% in control without pre-incubation of CPAE).

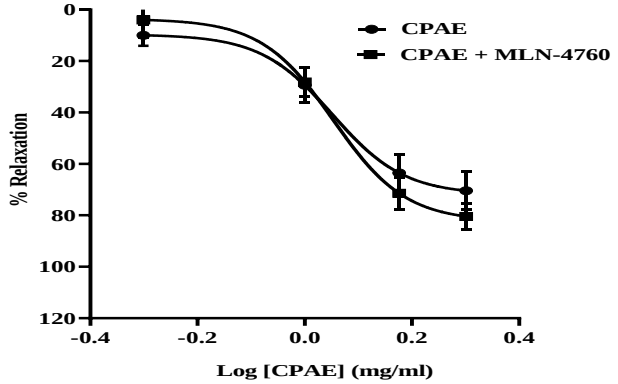

Figure 7: Effect of ACE-2 inhibition on the relaxation of aortic rings induced by the addition of cumulative doses of CPAE in the absence (circles) or in the presence (squares) of MLN-4760. Data represent mean ± SEM.

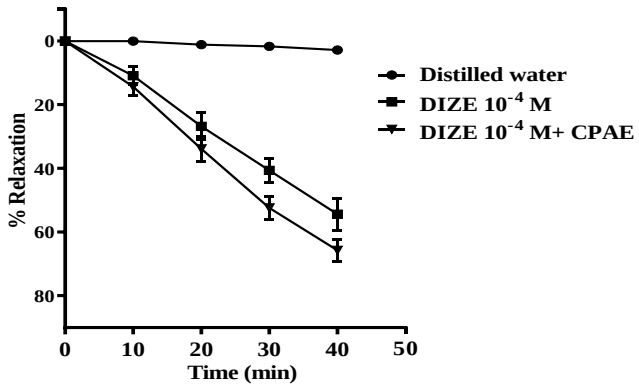

Figure 8: Effect of CPAE pre-incubation (0.375 mg/ml) on Diminazene-induced vasorelaxation in isolated aortic rings pre-contracted by EP. Data are shown as mean ± SEM.

ACE-2 constitutes a functional receptor that serves as the entry point for SARS-CoV-2 in human lung cells.[26] The researchers suggest that the potential inhibitory effects of ACE-2 could provide a viable strategy for the management and prevention of COVID-19.[27,28] Diminazene aceturate (DIZE) is known as a specific ACE-2 activator, it exerts its vasorelaxant effect via activation of ACE2/Ang-(1-7)/Mas axis[29], it was used to induce vasorelaxation in isolated aortic rings.[30] Effectively, the results of the present study showed that diminazene induced a significant vasodilation in the aortic rings, which aligns with previous findings.[29,31] The current experimental findings of the present study show that the pretreatment with CPAE did not inhibit DIZE-induced vasorelaxation in isolated aortic rings pre-contracted by EP, suggesting that CPAE has no inhibitory effect on ACE-2.

Conclusion

The present study demonstrates that the aqueous leaf extract of *Cistanche phelypaea* possess a potent antihypertensive effect in rats with a significant vasorelaxant activity on aortic rings by blocking Ca^{2+} mobilization via the inhibition of receptor-operated calcium channels. Indeed, this inhibition of extracellular calcium influx could explain the antihypertensive activity of this extract. On the other hand, the findings show that CPAE had no impact on angiotensin- converting enzyme-2. Nevertheless, additional investigations are required to validate the efficacy of this plant for treating hypertension, and to identify the bio active compound(s) responsible for these pharmacological effects.

Conflict of Interest

The authors declare no conflict of interest

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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