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RESEARCH ARTICLE

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Vasorelaxant effects of the edible flowers *Tagetes erecta* L. and its possible mechanism of action

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ABSTRACT

Context: *Tagetes erecta* is widely cultivated for its ornamental flowers and has traditionally been used as a diuretic and antihypertensive. However, its effects on blood pressure have not yet been studied.

Objective: To evaluate the vasorelaxant potential of ethanolic extract of *T. erecta* from two cultivars of edible flowers, yellow and orange.

Materials and Methods: The pharmacological effects of *T. erecta* extracts as vasorelaxant agents were evaluated using isolated rat aorta rings in an organ bath and by measuring the pharyngeal pumping rate in the *Caenorhabditis elegans* model.

Results: The extracts induced relaxation in endothelium-intact aortic rings pre-contracted with different agents. Vasorelaxant effect was attenuated by endothelial removal and by pretreatment with L-NAME or ODQ, but not by indomethacin. Statistically significant effects were observed only at low concentrations. Atropine and H-89 reduced the extract-induced response, whereas okadaic acid had no effect. In a calcium-free medium, the extracts reduced contractions induced by $CaCl_2$ and phenylephrine. Relaxation was significantly attenuated by iberiotoxin, glibenclamide, $BaCl_2$, and 4-aminopyridine, while apamin and TRAM-34 had mild effect. The extracts also significantly decreased the pharyngeal pumping rate in *C. elegans*.

Discussion and Conclusion: The extracts induced concentration-dependent vasorelaxation though both endothelium-dependent and -independent mechanisms. At low concentrations, relaxation was mediated by nitric oxide, while at higher concentrations it involved inhibition of intracellular Ca^{2+}, opening of K^+ channels, and activation of protein kinase A. In *C. elegans*, the extracts significantly reduced pharyngeal pumping. This study is the first to suggest that *T. erecta* could be beneficial in treating pathologies associated with endothelial dysfunction, such as hypertension.

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C. elegans; hypotensive; marigold; rat aorta; vasorelaxant effect

Introduction

Hypertension, as stated by the World Health Organization, is a significant contributor to premature mortality on a global scale, affecting an estimated 1.28 billion adults aged 30 and 79 years (WHO 2023). Hypertension, or high blood pressure, due to increased cardiac output and/or vascular resistance, is a serious disorder that significantly increases the risk of cardiovascular diseases, which remain leading causes of morbidity and mortality worldwide (Naghavi et al. 2015). Its pathophysiology is multifactorial, involving genetic predisposition, activation of the sympathetic nervous system, dysregulation of the renin-angiotensin-aldosterone system, and endothelial dysfunction (ED) (Konukoglu and Uzun 2017; Gallo et al. 2021). Furthermore, this pathology promotes a vicious cycle, as hypertension, along with other risk factors such as elevated LDL cholesterol, diabetes, smoking, reactive oxygen species, and inflammatory mechanisms, can activate and/or damage endothelial cells, impairing their multiple functions and exacerbating ED (Park and Park 2015; Gallo et al. 2021).

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The endothelium plays a fundamental role in the cardiovascular system, as it forms a mechanical and biological barrier between blood and vascular smooth muscle cells (VSMCs), while also regulating vascular tone, cell growth, and platelet aggregation. ED refers to the loss of the endothelium's ability to control vascular tone due to an imbalance between vasodilatory and vasoconstrictive factors, disruption of local hemostasis, and cell proliferation in the vessel wall. Therefore, ED promotes the development of various cardiovascular pathologies, such as atherosclerosis, thrombosis, and hypertension. ED is present in other conditions not directly related to cardiovascular diseases, such as diabetes, obesity, and aging (Konukoglu and Uzun 2017; Gallo et al. 2021).

Although there are numerous pharmacological treatments for high blood pressure, they are sometimes ineffective, leading to a high percentage of uncontrolled hypertension. Today, healthy lifestyle habits, such as a balanced diet and physical exercise, remain the primary strategies for prevention. Diets rich in vegetables and fruits contain large amounts of polyphenols and carotenoids, which exert antioxidant, anti-inflammatory, antihypertensive, antithrombotic, and anti-proliferative effects by improving endothelial function through multiple mechanisms in endothelial cells and/or VSMCs (Ciumărnean et al. 2020; Grosso et al. 2022; Abbasian et al. 2023). For example, these compounds can directly induce the release of vasodilatory factors, such as nitric oxide (NO), prostacyclin, or endothelium-derived relaxing factor, and/or inhibit vasoconstrictor factors, such as endothelin-1 and thromboxane. They can also inhibit phosphodiesterase enzymes, thereby preventing the degradation of cyclic nucleotides such as cGMP and cAMP, consequently increasing nucleotide-dependent protein kinases G and A (PKG and PKA). Additionally, these compounds can modulate calcium and potassium channels or inhibit vascular contraction pathways, such as protein kinase C (PKC). As a result, these mechanisms contribute to VSMC hyperpolarization and vasorelaxation, ultimately protecting endothelial function and the cardiovascular system (Ciumărnean et al. 2020; Grosso et al. 2022).

Edible flowers of *Tagetes erecta* L., a plant rich in phenolic compounds, have been traditionally used for centuries as a diuretic, hypotensive agent, and for the treatment of kidney, gastrointestinal, and skin disorders, as well as an analgesic (Mollik et al. 2010; Gopi et al. 2012; Michel et al. 2020). *T. erecta*, from the Asteraceae family, is native to Mexico, where it is commonly known as American marigold or Aztec marigold. Today, *T. erecta* is cultivated worldwide as an ornamental plant due to the yellow and orange coloration of its flowers and for the extraction of carotenoids, such as lutein (Manzoor et al. 2022). The phytochemical composition of different extracts of *T. erecta* has shown that this species is rich in phenolic acids, flavonoids, and pigments (Hegde et al. 2023; Núñez et al. 2023; 2025).

Previous studies have reported that *T. erecta* exhibits antioxidant, anti-inflammatory, antimicrobial, anti-hyperlipidemic, antidiabetic, and spasmolytic activities (Moliner et al. 2018; Ventura-Martínez et al. 2018; Núñez et al. 2023). Additionally, it has recently been demonstrated that *T. erecta* prevents the formation of advanced glycation end products (Moliner et al. 2018; Núñez et al. 2023), which are involved in inflammatory and oxidative processes that contribute to endothelial and vascular damage (Stirban et al. 2014). Ethnopharmacological studies have demonstrated that *T. erecta* exerts diuretic effects, increasing urine volume and sodium concentration, as well as the ability to inhibit the formation of calcium oxalate crystals (Zanovello et al. 2021). These pharmacological properties, along with its phytochemical composition, suggest that *T. erecta* may exert cardioprotective effects. However, its effects on the cardiovascular system have not yet been investigated. Therefore, the aim of this study was to evaluate the vascular effects of *T. erecta* extracts from two cultivars of this edible flower, yellow and orange, using isolated rat aorta rings, and to explore their mechanisms of action through myography by examining the pathways involved in the regulation of vascular tone. Additionally, the potential effect on heart rate was assessed using an *in vivo C. elegans* model, taking advantage of functional similarity between the human heart and the pharynx of these nematodes (Srinivasan et al. 2023).

Materials and methods

Reagents and chemicals

Acetylcholine (ACh, Ref: A6625); angiotensin II (Ang II, Ref: A9525); apamin (AP, Ref: A1289); atropine (Ref: Y0000878); barium chloride dihydrate ($BaCl_2$, Ref: 217565); glibenclamide (Glib, Ref:

G0639); H-89 dihydrochloride hydrate (H-89, Ref: B1427); iberiotoxin (IbTX, Ref: I5904); indomethacin (Ref: I8280); Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, Ref: 483125-M); okadaic acid (Ref: 495604); 5-hydroxytryptamine (5-HT, Ref: 14927); phenylephrine (PE, Ref: P6126); verapamil (V, Ref: 676777); 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, Ref: O3636); and 4-aminopyridine (4-AP, Ref: 275875) were obtained from Sigma (Madrid, Spain). TRAM-34 (Ref: T6700) was purchased from Tocris (Madrid, Spain). All chemicals were of analytical grade. AP was diluted in acetic acid. Glib, H-89 and TRAM-34 were prepared in dimethyl sulfoxide (DMSO). The final concentrations of DMSO and acetic acid did not exceed 0.1% and had no direct effect on tissue contractility. All other chemicals were dissolved in distilled water.

The composition of the buffers used was as follows: Krebs buffer (in millimolar): NaCl 120 (Ref: S9888), KCl 4.7 (Ref: P9541), $CaCl_2$ 2.4 (Ref: C5670), $MgSO_4$ 1.2 (Ref: 746452), $NaHCO_3$ 24.5 (Ref: S5761), KH_2PO_4 1 (Ref: P0662), and glucose 5.6 (Ref: G8270). Calcium-free Krebs: NaCl 120 (Ref: S9888), KCl 4.7 (Ref: P9541), $CaCl_2$ 0 (Ref: C5670), $MgSO_4$ 1.2 (Ref: 746452), $NaHCO_3$ 24.5 (Ref: S5761), KH_2PO_4 1 (Ref: P0662), glucose 5.6 (Ref: G8270), and ethylene glycol tetraacetic acid (EGTA) 1 (Ref: E8145). Calcium-free, high K^+ Krebs ($[K^+]_o$=50 mM). All buffers were adjusted to pH 7.4. All compounds were obtained from Sigma (Madrid, Spain).

Plant material and its phytochemical composition

Yellow (Ref: 007–2022) and orange (Ref: 008–2022) cultivars of edible *Tagetes erecta* flowers were acquired from Innoflower SL (Batch 710/2022) after harvesting in April 2022. Fresh flowers were cut into small pieces, and the extracts were prepared using ultrasound-assisted extraction (UAE), a non-conventional extraction method, with ethanol as the solvent for 4 cycles of 35 min. The solvent was removed using a rotary evaporator, and the final extracts, Yellow Tagetes (YT) and Orange Tagetes (OT), were stored at −20 °C in the dark until use. Phytochemical characterization and the quantification of bioactive compounds in *T. erecta* extracts by chromatographic techniques was previously published (Núñez et al. 2025). In our extracts, 26 polyphenols were detected and quantified by HPLC-MS/MS, with ellagic, gallic, and vanillic acids the most abundant compounds among the phenolic acids, while quercetin, isoquercitrin, isorhamnetin, and kaempferol were the most prevalent flavonoids. Pigments such as lutein, β-carotenoids, and tocopherols were also detected using HPLC-DAD-FLD (Núñez et al. 2025).

Ex vivo assay

Animal tissue

Animal research is a fundamental part of thousands of original publications each year, generating significant advances across many research fields. However, it involves the sacrifice of a large number of animals annually. For this reason, in this study, only surplus tissue was used.

Aortic tissue was obtained from surplus material of healthy male Wistar rats (n = 40, weight range: 200–250 g) used in previous experiments. No animals were sacrificed specifically for this study. All procedures complied with ethical standards, the institution's 3 R policy, and relevant animal welfare regulations, including the Spanish Animal Protection Policy RD 53/2013, which aligns with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. Animals were housed under standard laboratory conditions with a standard diet and unlimited access to water. Euthanasia in the original experiments was performed using pentobarbital sodium (60 mg/kg i.p.) followed by cervical dislocation (Valero et al. 2022). Tissue was collected as part of a previous project approved by the Ethics Committee for Animal Experimentation of the University of Zaragoza (Spain), reference number PI66/17.

Preparation of aortic rings and isometric myography

Myography experiments were performed as described by Alda et al. (2009). After cervical dislocation, the thoracic aorta was carefully removed and placed into ice-cold Krebs solution, from which fat and

connective tissue were cleaned. The aorta was then cut into 3 mm rings. Rat aortic rings were individually connected to an isometric force transducer (Pioden UF1, Graham Bell House, Canterbury, UK) for tension measurement and were suspended in an organ bath containing 5 mL of Krebs buffer, maintained at 37 °C and continuously gassed with a mixture of 95% O_2 and 5% CO_2 (3 bar of pressure and 1 L/min of flow). Tension data were recorded and digitized at a sampling rate of 0.5 Hz using an ED410 e-corder 410 data acquisition system with eDAQ Chart v5.5 software (eDAQ, Cibertec, Madrid, Spain).

At the beginning of each experiment, an initial tension of 1 g was applied to the preparations to achieve spontaneous tone, and rings were allowed to equilibrate for 60 min, with the bath buffer being changed every 20 min during the equilibrium period.

Experimental protocols

Effects of extracts on contractile responses induced by contractile agents. After stabilization of spontaneous tone, PE (10^{-6} M), KCl (80 mM), and 5-HT (10^{-4} M) were used to induce sustained contractions in endothelium-intact aortic rings (blood vessels with a complete and functional endothelial layer). After reaching a plateau phase, increasing concentrations of YT and OT extracts (0.01, 0.03, 0.1, 0.3, 0.5, 1, and 2 mg/mL) were added every 15 min, and cumulative concentration-response curves for the extracts were performed. The vasorelaxant effect of the extracts was determined by comparing the pre-contraction value relative to the basal tone (in the absence of the contractile agent), which was taken as 100%, and calculating the percentage of change.

Role of the endothelium in the vasorelaxant effect of T. erecta extracts. To measure the relaxing response specifically mediated by the endothelium, a series of experiments were carried out. The endothelium was mechanically disrupted by gently rubbing the intimal surface of the aorta with a small wooden stick (endothelium-denuded). The absence of a functional endothelium was confirmed by adding ACh (10^{-4} M) to the PE-pre-contracted rings (10^{-6} M). Aortic rings that relaxed by only 10-15% were considered endothelium-denuded. The protocol used to study the role of endothelium in the vasorelaxant effect of the extracts was the same as described in the previous section.

To further evaluate the endothelium-independent vasorelaxation induced by *T. erecta* extracts, the release of endothelium-derived factors was chemically inhibited by pre-incubating the rings for 20 min with the NO-synthase blocker L-NAME (10^{-5} M) and the cyclooxygenase blocker indomethacin (10^{-5} M) before the addition of PE (endothelium-denuded). After sustained contraction, a cumulative concentration-response curve for the extract was performed.

Vasorelaxant response obtained with *T. erecta* extract alone (control) was compared with the response obtained with *T. erecta* extracts after pretreatment with the different substances.

Role of secondary messengers in the vasorelaxant effect of the extracts. Secondary messengers such as soluble guanylate cyclase (sGC) or protein kinase A (PKA) were investigated in the response to YT and OT extracts. Endothelium-intact aortic rings were pre-incubated with ODQ (10^{-5} M), a potent and selective inhibitor of nitric oxide (NO)-sensitive guanylyl cyclase, or H-89 (2×10^{-7} M), a PKA inhibitor, for 20 min before the addition of PE (10^{-6} M). Once the contraction was stable, YT or OT extracts (0.3, 1, and 2 mg/mL) were added for 15 min. The relaxant effect was compared with the response obtained from YT or OT extracts alone (control).

Effects of extracts pretreatment on PE- and ang II-induced contractions. The effect of extracts pretreatment on the contractile responses to PE and Ang II was studied. Endothelium-intact aortic rings were pre-incubated with either solvent, YT or OT extracts (0.3 and 1 mg/mL), or V (10^{-6} M) for 20 min. Cumulative concentration-response curves were then generated for PE (10^{-9}–10^{-5} M) and Ang II (10^{-10}–10^{-6} M). The contractile responses induced by PE and Ang II were calculated with respect to the basal line. The change in tension due to the contractile agents was compared in the absence (control) or presence of the extract or verapamil.

Role of calcium in the effects of T. erecta extracts. To investigate whether calcium is involved in the relaxing response induced by the extracts, the following tests were carried out. First, we studied whether the response produced by the extracts involved a decrease in calcium influx from the extracellular medium. To this end, after an initial incubation period in Krebs buffer, the medium was replaced by Ca^{2+}-free Krebs for 20 min, followed by replacement with Ca^{2+}-free, high-K^+ buffer. Endothelium-intact rings were then pre-incubated for 20 min with solvent, YT or OT extracts (0.3 and 1 mg/mL) or verapamil (10^{-6} M). After this, cumulative concentration-response curves for $CaCl_2$ (10^{-5}–10^{-2} M) were constructed. The responses to $CaCl_2$ were compared in the absence (control) or presence of the extracts or verapamil.

Second, to study whether the response produced by the extract involved inhibition of calcium release from the sarcoplasmic reticulum, we examined the effect of the extracts on the PE-induced contractions in a calcium-free medium. Endothelium-intact rings were pre-incubated for 20 min with solvent, YT or OT extracts (0.3 and 1 mg/mL), and then PE 10^{-6} M was added. Contractions induced by PE were calculated as the change in tension relative to the basal line, and the responses were compared in the absence (control) and presence of the pretreatment extracts.

Effect of potassium channels on the aortic response to T. erecta extracts. To investigate the involvement of potassium channels in the vasorelaxant responses to YT and OT extracts, endothelium-intact aortic rings were pre-incubated with TRAM-34, a selective inhibitor of intermediate-conductance Ca^{2+}-activated K^+ channel; AP (10^{-6} M), a selective inhibitor of small-conductance Ca^{2+}-activated K^+ channel; IbTX (3×10^{-8} M), a selective inhibitor of big-conductance Ca^{2+}-activated K^+ channel; Glib (10^{-5} M), a nonspecific ATP-sensitive K^+ channel blocker; $BaCl_2$ (3×10^{-5} M), an inwardly rectifying K^+ channel blocker; and 4-AP (10^{-3} M) a voltage dependent K^+ channel blocker, 20 min before the addition of PE (10^{-6} M). After stabilization of the contraction, *T. erecta* extracts (0.3 and 1 mg/mL) were added for 15 min. The relaxant effect was compared to the response obtained from *T. erecta* extracts alone (control).

Effect of other agents on the response to T. erecta extracts. In other experiments, we studied whether the muscarinic receptors and the smooth muscle contractile apparatus were involved in the relaxing response to the extracts. To do this, we incubated the endothelium-intact rings with atropine (10^{-6} M), a competitive antagonist of muscarinic receptor, or okadaic acid (10^{-6} M), a potent inhibitor of myosin light chain phosphatase, for 20 min. Then, the rings were pre-contracted with PE (10^{-6} M), and dose-response curves were obtained by adding YT and OT extracts. The relaxing effect was compared with the response obtained from the *T. erecta* extracts alone (control).

In vivo assay

Strains and maintenance conditions

Caenorhabditis elegans strain N2, Bristol (wild type; Ref: USJ-Cepa N2), was provided by the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN, USA). Nematodes were grown and maintained on nematode growth medium (NGM) at 20 °C, using *Escherichia coli* OP50 (Ref: USJ-*E.coli* OP50) bacteria as a food source, also obtained from the CGC. For all experiments, synchronized N2 worms were obtained using an alkali-bleaching method.

Pharyngeal pumping

Following previous protocols, synchronized L1 *C. elegans* were grown at 20 °C until reaching the L4 stage and then exposed to the following conditions: control (NGM only), obese model (5% glucose), and treatment (250 µg/mL extract + 5% glucose). Videos of 30 s were recorded on days 2 and 6 for each worm (at least 10 worms per condition), and pharyngeal pumping was measured by counting the rhythmic contractions of the pharynx during that time-lapse. Each worm was counted thrice, and the data were averaged. This assay was repeated independently three times.

Statistical analysis

Data values are expressed as mean ± standard error of the mean (SEM). The Shapiro–Wilk test was performed to assess whether the data followed a normal distribution. According to the results, statistical comparisons between experimental groups were performed by one-way analysis of variance (ANOVA) followed by the Dunnett test (when a parametric distribution was observed), or the Kruskal-Wallis test followed by the Dunn test (when a nonparametric distribution was observed). For pairwise comparisons, either a paired two-tailed Student's t-test (parametric data) or the Mann–Whitney U test (non-parametric data) was applied. A two-way ANOVA was performed to evaluate both the interaction between the two variables and the main effects of each factor. All analyses were performed using GraphPad Prism 6. A $p < 0.05$ was considered statistically significant. The concentration of the compound that inhibited 50% of the maximal contraction (EC_{50}) was calculated as the geometric mean with 95% confidence intervals (CI) for the concentration-response curve experiments. ToupView was used to take videos for the assays involving *C. elegans*.

Results

Ex vivo assays

Vasorelaxant effects of T. erecta extracts on phenylephrine, 5-hydroxytryptamine, and KCl-induced contractions

The effect of Yellow Tagetes (YT) and Orange Tagetes (OT) extracts (0.01–2 mg/mL) on endothelium-intact aortic rings pre-contracted by phenylephrine (PE, 10^{-6} M), 5-hydroxytryptamine (5-HT, 10^{-5} M), and KCl (80 mM) was studied. The vasoconstrictor agents produced a contractile response in the vascular smooth muscle, maintained throughout the duration of the assay.

The *T. erecta* extracts induced a concentration-dependent vasorelaxation in PE-, 5-HT-, or KCl-pre-contracted rings (Figure 1(a,b)). Cumulative addition of the vehicle for the extracts had no effect on vascular tone with any of the vasocontractile agents.

As shown in Figure 1, the extracts induced a concentration-dependent vasorelaxation in the PE-pre-contracted rings, with EC_{50} values of 0.74 mg/mL (0.66–0.82, 95% CI) and 1.02 mg/mL (0.91–1.15, 95% CI) for the YT and OT extracts, respectively (Figure 1(c)). In 5-HT-pre-contracted rings, the EC_{50} values for the YT and OT extracts were 1.09 mg/mL (0.92–1.27, 95% CI) and 1.31 mg/mL (1.12–1.52, 95% CI), respectively (Figure 1(d)). Meanwhile, the EC_{50} values of YT and OT extracts in the KCl-pre-contracted rings were 2.16 mg/mL and 2.14 mg/mL, respectively (Figure 1(e)). As can be seen, the YT extract showed a greater relaxing response than the OT extract.

Although *T. erecta* showed a vasorelaxant effect on the contractions caused by all contractile agents, the relaxation produced at the highest dose of extract was significantly lower in the KCl-pre-contracted rings compared with those pre-contracted with 5-HT or PE (YT extract: 47.5% ± 4.0 < 69.1% ± 6.0 < 83.4% ± 4.2; OT extract: 48.8% ± 5.5 < 64.2% ± 6.2 < 69.7% ± 4.1, respectively). These results suggest that the relaxing response of the extracts could be related to the opening of potassium channels.

As the greatest relaxation induced by the extracts was obtained in PE-pre-contracted rings, PE was used as the contractile agent in the following experiments to investigate its possible mechanism of action.

Effect of the endothelium on T. erecta extract-induced vasorelaxation

To better understand the role of the endothelium in the relaxation produced by the *T. erecta* extracts, the endothelium was either physically removed to obtain endothelium-denuded rings or chemically inhibited in endothelium-intact aortic rings by blocking endothelium-derived factors.

As shown in Figure 2(a,b), YT and OT extracts also produced a concentration-dependent vasorelaxant response in endothelium-denuded aortic rings pre-contracted with PE (10^{-6} M). However, this response was lower, although not significantly, compared with that obtained in endothelium-intact

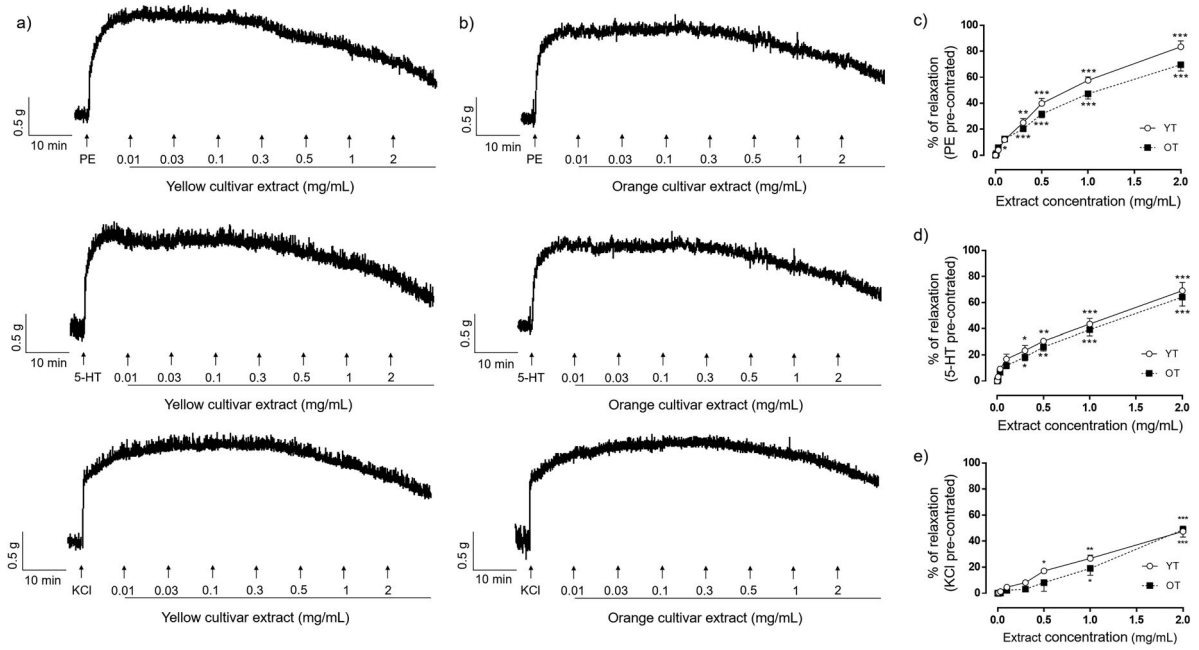

Figure 1. Representative recordings showing the precontractions evoked in endothelium-intact rat aortic rings by phenylephrine (PE, 10^{-6} M), 5-hydroxytryptamine (5-HT, 10^{-6} M), and KCl (80 mM), and the effects induced by (a) yellow cultivar extracts and (b) orange cultivar extracts (0.01–2 mg/mL) on these contractions. Relaxant effect of different concentrations of two *T. erecta* cultivar extracts (mg/mL), yellow (YT) and orange (OT) flowers, applied to rat aortic rings previously pre-contracted with (c) PE (d) 5-HT, and (e) KCl. Data points are presented as mean ± SEM ($n = 6$–12). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. control (basal tone). Statistical analysis: one-way ANOVA with dunnett's test (for parametric data) or kruskal–wallis with dunn's test (for non-parametric data). (c) YT 0.3 mg/mL: $p = 0.0330$ (*); YT 0.5 mg/mL: $p = 0.0087$ (**); YT 1 and 2 mg/mL: $p \leq 0.001$ (***). OT 0.1 mg/mL: $p = 0.0249$ (*); OT 0.3, 0.5, 1, and 2 mg/mL: $p \leq 0.001$ (***).(d) YT 0.3 mg/mL: $p = 0.0210$ (*); YT 0.5 mg/mL: $p = 0.0049$ (**); YT 1 and 2 mg/mL: $p \leq 0.001$ (***). OT 0.3 mg/mL: $p = 0.0435$ (*); OT 0.5 mg/mL: $p = 0.003$ (**); OT 1 and 2 mg/mL: $p \leq 0.001$ (***).(e) YT 0.5 mg/mL: $p = 0.0189$ (*); YT 1 mg/mL: $p = 0.002$ (**) and YT 2 mg/mL: $p \leq 0.001$ (***). OT 1 mg/mL: $p = 0.0486$ (*); and OT 2 mg/mL: $p \leq 0.001$ (***).

rings (EC_{50} values of 0.95 mg/mL (0.88–1.03, 95% CI) vs. 0.74 mg/mL (0.66–0.82, 95% CI) for YT extract, and EC_{50} values of 1.28 mg/mL (1.14–1.42, 95% CI) vs. 1.02 mg/mL (0.91–1.15, 95% CI) for OT extract in rings with and without endothelium, respectively).

At low doses of *T. erecta* extracts, the vasorelaxant response was lower in endothelium-denuded rings than in endothelium-intact rings. However, at the highest dose, the maximal relaxant effect of extracts was similar in both types of rings. For the yellow extract, the response was 83.4% ± 4.2 and 83.3% ± 4.5 in endothelium-intact and endothelium-denuded aortic rings, respectively. For the orange extract, the values were lower: 69.7% ± 4.1 and 68.6% ± 4.0, respectively.

To further evaluate the effects of *T. erecta* on endothelium-derived vasorelaxant factors, endothelium-intact aortic rings were pre-incubated with L-NAME (10^{-5} M), a nitric oxide synthase inhibitor, and indomethacin (10^{-5} M), a nonselective cyclooxygenase inhibitor. Pretreatment with L-NAME significantly reduced the relaxing response of the extracts at low doses (0.1 and 0.3 mg/mL). However, L-NAME did not modify the response to high doses of the extracts. Pretreatment with indomethacin had no effect on *T. erecta* extracts-induced relaxation of rings contracted by PE (10^{-6} M) (Figure 2(c,d)).

These results suggest that the vasorelaxant effect of *T. erecta* is dependent, but not exclusively, on the release of endothelium-derived NO mediators.

Effect of soluble guanylyl cyclase on T. erecta extract-induced vasorelaxation

We studied whether *T. erecta*-induced vasodilation was related to cGMP signaling pathways. To this end, endothelial-intact rings were pre-incubated with ODQ (10^{-5} M), a potent and selective soluble

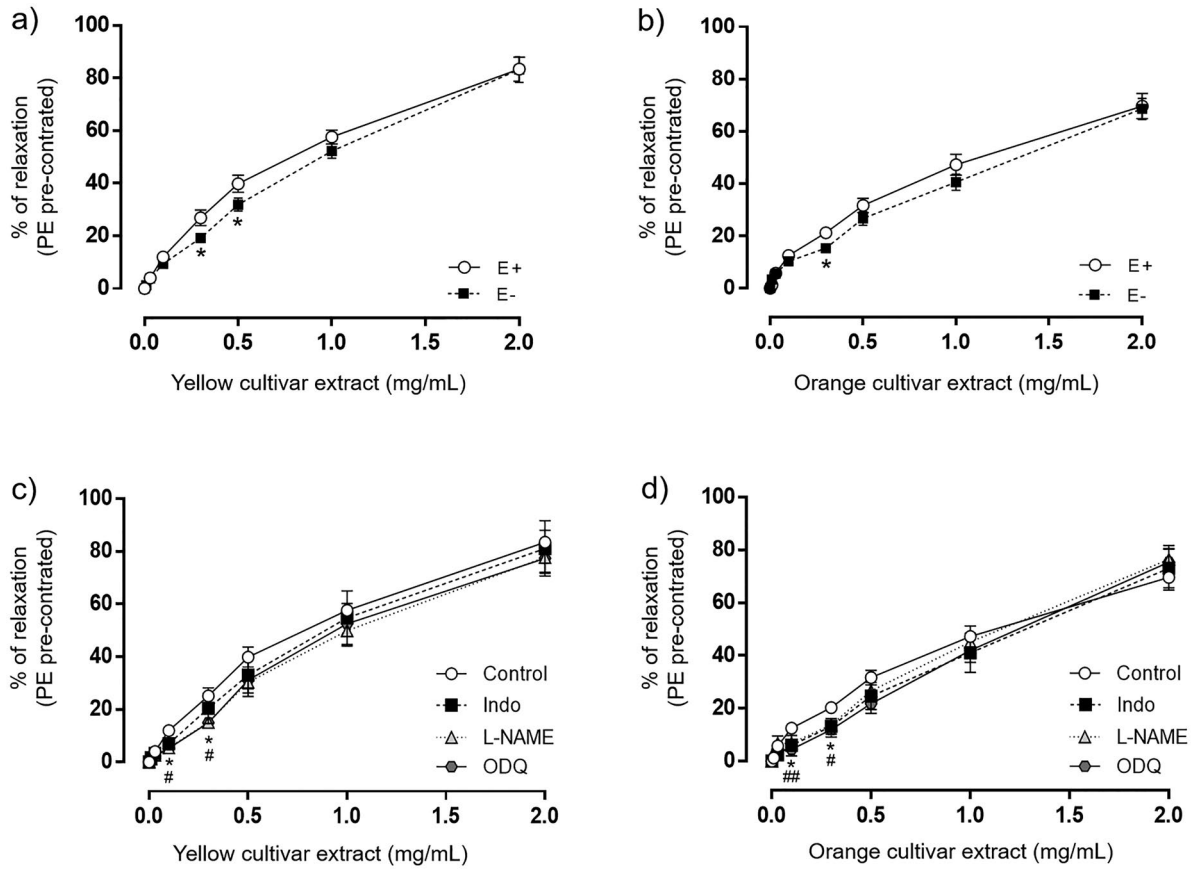

Figure 2. Relaxant effects of different concentrations of (a) yellow cultivar extract and (b) orange cultivar extract on phenylephrine (PE, 10^{-6} M)-induced contractions in isolated rat aortic rings with endothelium (E+) and without endothelium (E–). data points are presented as mean ± SEM ($n = 6–8$). *$p < 0.05$ vs. control (E+). statistical analysis: paired two-tailed student's t-test. (a) YT 0.3 mg/mL: $p = 0.0274$ (*); YT 0.5 mg/mL: $p = 0.0350$ (*).(b) OT 0.3 mg/mL: $p = 0.0161$ (*). relaxant effects of different concentrations of (c) yellow cultivar extract and (d) orange cultivar extract in the absence (control) and presence of indomethacin (indo, 10^{-5} M), L-NAME (10^{-5} M), and ODQ (10^{-5} M) on phenylephrine-induced contractions in endothelium-intact rings. Data points are presented as mean ± SEM ($n = 6–8$). *$p < 0.05$ L-NAME vs. control (extract alone) and #$p < 0.05$, ##$p < 0.01$ ODQ vs. control (extract alone). Statistical analysis: paired two-tailed student's t-test or the mann–whitney U test (non-parametric data). (c) YT 0.1 mg/mL: $p = 0.0173$ (*) and $p = 0.0289$ (#); YT 0.3 mg/mL: $p = 0.0210$ (*) and $p = 0.0275$ (#); OT 0.1 mg/mL: $p = 0.0469$ (*) and $p = 0.0048$ (##). (d) OT 0.3 mg/mL: $p = 0.0326$ (*) and $p = 0.0194$ (#).

guanylyl cyclase (sGC) inhibitor. Afterwards, the vasorelaxant responses of the extracts were studied in aortic rings contracted with PE 10^{-6} M (control). Pretreatment with ODQ significantly inhibited the relaxation induced by the extracts at low doses (0.1 and 0.3 mg/mL), but the inhibitory effect was lost at higher concentrations (Figure 2(c,d)). These results demonstrate that the NO/cGMP pathway partially contributes to the relaxation induced by *T. erecta* extracts.

Effect of atropine on T. erecta extract-induced vasorelaxation

To investigate the effect of *T. erecta* extracts on muscarinic receptors, endothelium-intact aortic rings were pre-incubated with atropine (10^{-6} M), a nonselective competitive muscarinic receptor antagonist, before pre-contraction with PE and the addition of extracts. As shown in Table 1, incubation with atropine non-significantly attenuated *T. erecta* extracts-induced relaxation of endothelium-intact aortic rings, except at 0.1 and 0.3 mg/mL. The EC_{50} values obtained after pre-incubation with atropine were 1.11 mg/mL (0.86–1.38, 95% CI) for YT extract and 1.39 mg/mL (1.13–1.76, 95% CI) for OT extract. These results demonstrate that muscarinic cholinergic receptors partially contribute to the relaxation induced by *T. erecta* extracts.

Effect of pre-incubation with T. erecta extracts on the contractile response to phenylephrine and angiotensin II

The effects of *T. erecta* extracts on the contractile response to PE and angiotensin II (Ang II) were studied. Endothelium-intact aortic rings were pre-incubated with solvent, YT and OT extracts at concentrations of 0.3 and 1 mg/mL, and verapamil (V, 10^{-6} M), an L-type calcium channel antagonist. Cumulative concentration-response curves for PE (10^{-9}–10^{-6} M) and Ang II (10^{-10}–10^{-6} M) were then constructed.

PE and Ang II induced concentration-dependent contractions in rings pre-incubated with solvent (control). Pretreatment with the extracts or V shifted the contraction curves to the right and downward compared with the control (Figure 3).

Specifically, pre-incubation with YT and OT extracts at 0.3 and 1 mg/mL reduced the contraction induced by PE at 10^{-6} M by 30.9% and 48.3%, and 25.8% and 59.8%, respectively, compared with the control. V reduced the contractile response to PE in a similar manner to the highest dose of extract (58.1%) (Figure 3(a,b).

As shown in Figure 3(c,d), the extracts at a dose of 0.3 mg/mL attenuated the contraction induced by the highest concentration of Ang II in a manner similar to V (44.9% for YT extract and 42.3% for OT extract vs. 49.6% for V). Meanwhile, the inhibition produced by the extracts at 1 mg/mL was stronger than the effect produced by V (80.1% for YT extract and 65.9% for OT extract vs. 49.6% for V).

Effect of calcium on T. erecta extract-induced vasorelaxation

To understand the role of calcium in the vasorelaxation induced by *T. erecta* extracts, the effects of extracellular calcium influx and calcium release from the sarcoplasmic reticulum were evaluated.

To evaluate the role of extracellular calcium, endothelium-intact aortic rings were pre-incubated in a Ca^{2+}-free, high-K^+-buffer with solvent, YT, or OT extracts (0.3 or 1 mg/mL). After the incubation period, cumulative additions of $CaCl_2$ were performed. In rings pre-incubated with the solvent (control), $CaCl_2$ induced progressively increasing tension. Treatment with the extracts significantly attenuated the $CaCl_2$-induced vasoconstriction in a concentration-dependent manner in the high-K^+ depolarizing solution. The maximum response to $CaCl_2$ was reduced by YT extract by 31.2% and 54.4%, and by the OT extract by 25% and 57%, at 0.3 and 1 mg/mL, respectively (Figure 4(a,b)).

To investigate whether intracellular calcium release was involved in the vasorelaxant response of *T. erecta* extracts, endothelium-intact aortic rings were pre-incubated with a Ca^{2+}-free Krebs solution for 20 min. Then, the rings were pre-incubated with solvent or *T. erecta* extracts (0.3 and 1 mg/mL) before the addition of PE (10^{-6} M). As shown in Figure 4(c), in a calcium-free medium, the contractile response to PE was lower than in a medium with calcium ($0.38\,g \pm 0.1$ vs. $0.79\,g \pm 0.1$). This response was due only to calcium released from the sarcoplasmic reticulum. The *T. erecta* extracts, in a concentration-dependent manner, reduced the contractile response to PE compared to the control (YT extract by 67.2% and 88.1% at 0.3 and 1 mg/mL, respectively, and OT extract by 71.7% and 75.9% at 0.3 and 1 mg/mL, respectively). These findings suggest inhibition of calcium entry from the extracellular medium and imply the importance of the IP_3 signaling pathway in the vasorelaxant response induced by *T. erecta*.

Table 1. Percentage of relaxation induced by yellow (YT) and orange (OT) cultivar extracts in the absence and presence of atropine (A, 10^{-6} M) on phenylephrine-induced contractions in endothelium-intact aortic rings.

mg/mL	YT	A + YT	OT	A + OT
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.01	0.4 ± 0.9	1.1 ± 0.6	1.2 ± 0.5	1.7 ± 0.6
0.03	4.0 ± 1.9	2.9 ± 1.7	5.8 ± 0.8	4.5 ± 1.0
0.1	12.0 ± 2.1	5.0 ± 1.7*	12.5 ± 1.2	9.4 ± 1.9
0.3	25.2 ± 3.0	14.6 ± 6.2*	20.3 ± 1.7	11.4 ± 3.8*
0.5	39.8 ± 3.7	27.0 ± 9.2	31.7 ± 2.7	21.8 ± 5.3
1	57.6 ± 2.6	47.8 ± 7.0	47.2 ± 3.9	38.2 ± 6.6
2	83.4 ± 4.2	70.6 ± 6.2	69.7 ± 4.1	59.5 ± 5.9

Data points are presented as mean ± SEM ($n = 8$–12). *$p < 0.05$ vs. extract alone. Statistical analysis: paired two-tailed Student's t-test (YT 0.1 mg/ml, $p = 0.0446$; YT 0.3 mg/ml, $p = 0.0446$; and OT 0.3 mg/mL, $p = 0.037$ (*).

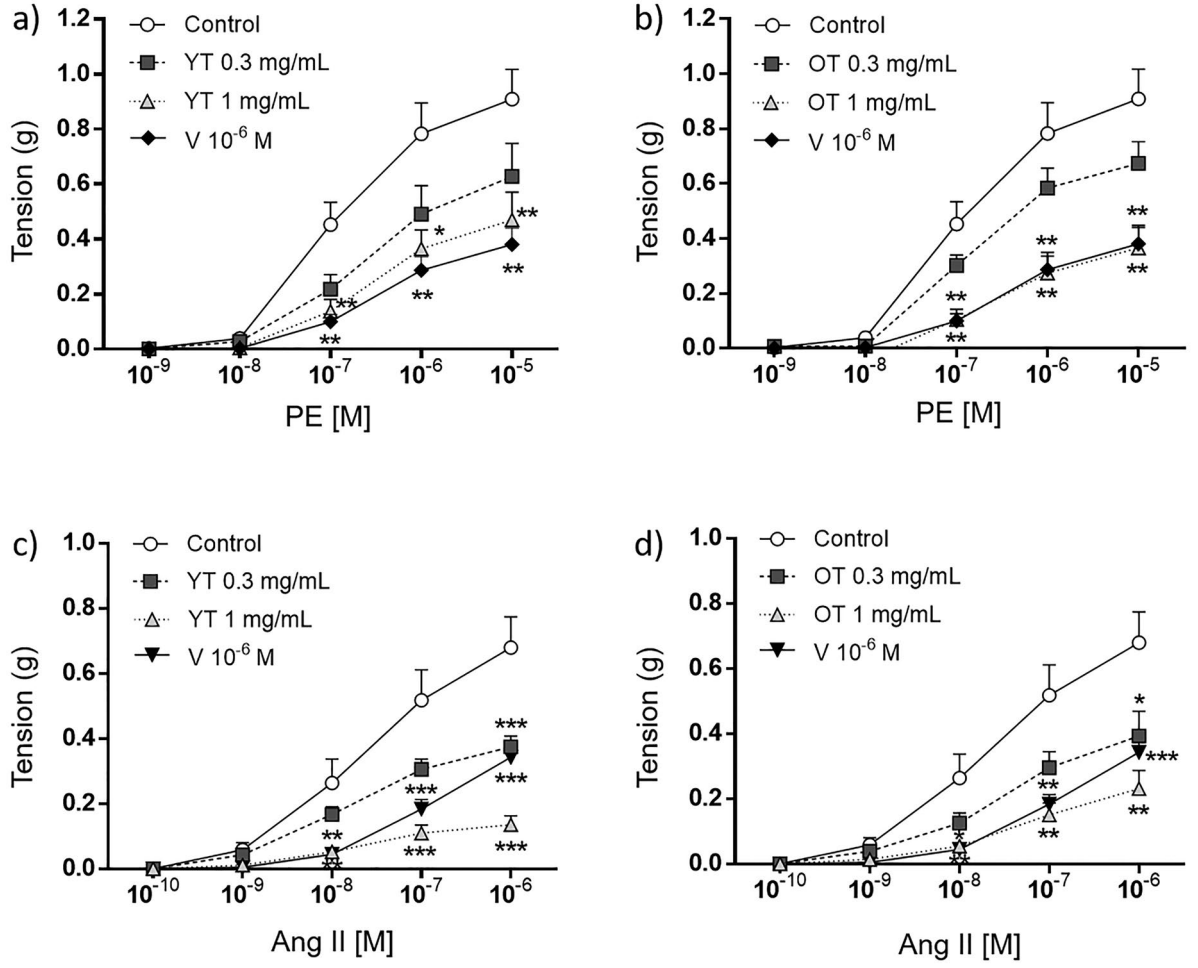

Figure 3. Effect of pre-incubation with *T. erecta* extracts, yellow cultivar (YT) (a and c) and orange cultivar (OT) extracts (b and d), and verapamil (V) on tension induced by different concentrations of phenylephrine (PE, 10^{-9}–10^{-5} M) (a and b) and angiotensin II (Ang II, 10^{-10}–10^{-6} M) (c and d), in a calcium-containing medium, in endothelium-intact aortic rings. Data points are presented as mean ± SEM ($n = 6$–8). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. control (solvent alone). Statistical analysis: paired two-tailed student's t-test. (a) YT 1 mg/mL: $p = 0.0056$ (**) for PE 10^{-7} M; $p = 0.0165$ (*) for PE 10^{-6} M; $p = 0.0012$ (**) for PE 10^{-5} M. (b) OT 1 mg/mL: $p = 0.008$ (**) for PE 10^{-7} M; $p = 0.003$ (*) for PE 10^{-6} M; $p = 0.0012$ (**) for PE 10^{-5} M. V 10^{-6} M: $p = 0.0024$ (**) for PE 10^{-7} M; $p = 0.0015$ (**) for PE 10^{-6} M; $p = 0.0018$ (**) for PE 10^{-5} M. (c) YT 0.3 mg/mL: $p = 0.001$ (***) for Ang II 10^{-6} M; YT 1 mg/mL: $p = 0.0091$ (**) for Ang II 10^{-8} M; $p = 0.0005$ (***) for Ang II 10^{-7} M; $p = 0.0001$ (***) for Ang II 10^{-6} M. (d) OT 1 mg/mL: $p = 0.0142$ (**) for Ang II 10^{-8} M; $p = 0.0026$ (**) for Ang II 10^{-7} M; $p = 0.003$ (**) for Ang II 10^{-6} M. V 10^{-6}M: $p = 0.0019$ (**) for Ang II 10^{-8} M; $p = 0.0008$ (***) for Ang II 10^{-7} M; $p = 0.0003$ (***) for Ang II 10^{-6} M).

Effect of potassium channels inhibitors on T. erecta extract-induced vasorelaxation

Different potassium channel blockers were used to investigate the role of potassium channels in the vasorelaxant response induced by *T. erecta* extracts. For this purpose, endothelium-intact aortic rings were pre-incubated with potassium channel blockers, such as apamin (AP, 10^{-6} M), TRAM-34 (10^{-6} M) and iberiotoxin (IbTX, 3×10^{-8} M), selective inhibitors of calcium-activated potassium channels of either small (SK_{Ca}), intermediate (IK_{Ca}) and big-conductance (BK_{Ca}) respectively. Additionally, glibenclamide (Glib, 10^{-5} M), an inhibitor of ATP-sensitive potassium channels (K_{ATP}); barium chloride ($BaCl_2$, 3×10^{-5} M), an inhibitor of the inward rectifier potassium channels (K_{IR}); and 4-aminopyridine (4-AP, 10^{-3} M), an inhibitor of the voltage dependent potassium channel (K_V), were also used.

The vasorelaxant effect of YT extract on endothelium-intact aortic rings pre-contracted with PE was reduced, although not significantly, after incubation with apamin and TRAM-34 at all three

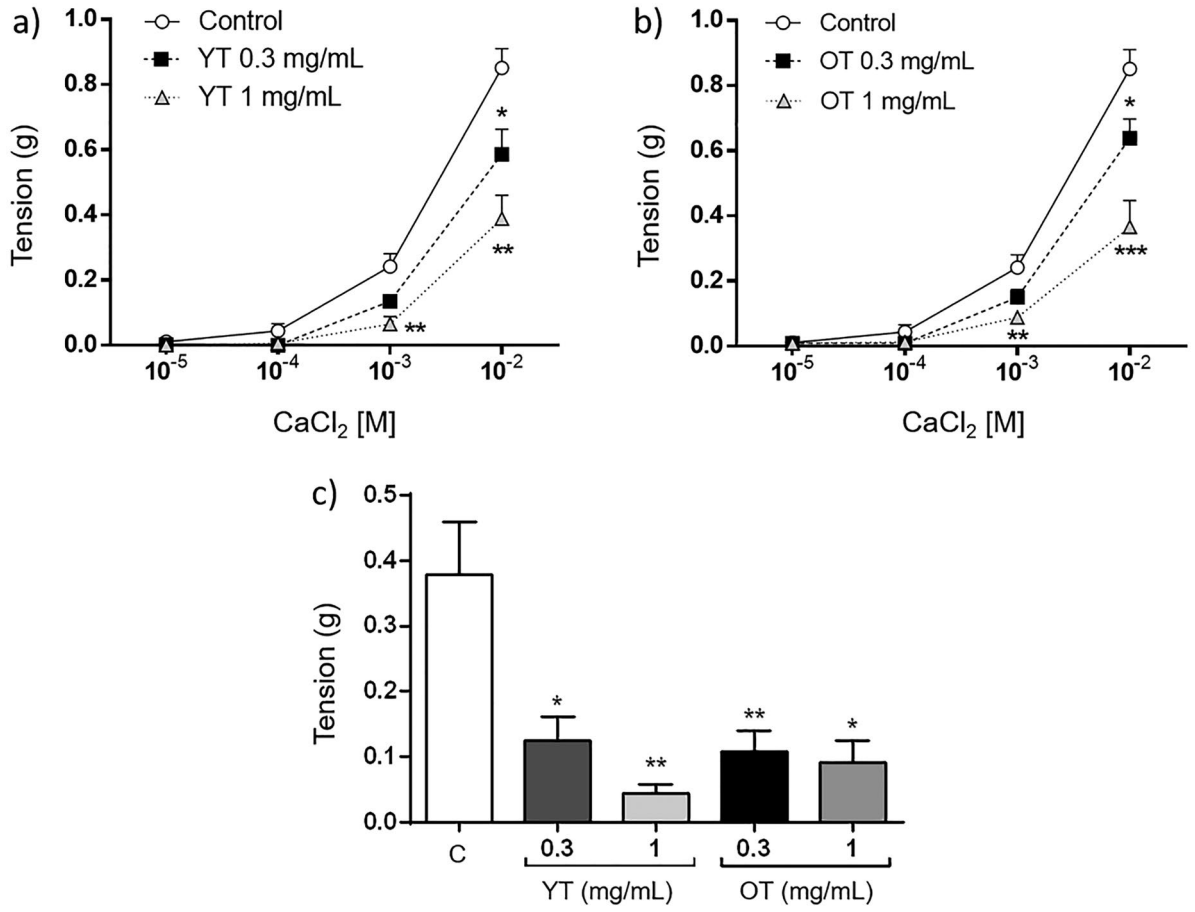

Figure 4. Effect of pre-incubation with solvent, (a) yellow cultivar (YT) and (b) orange cultivar (OT) extracts (0.3 and 1 mg/mL) on contraction induced by $CaCl_2$ (10^{-5}–10^{-2} M) in endothelium-intact aortic rings in a calcium-free medium. Data are presented as mean ± SEM ($n = 6$–8). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. control (solvent alone). Statistical analysis: paired two-tailed student's t-test. (a) YT 0.3 mg/mL: $p = 0.0193$ (*) for $CaCl_2$ 10^{-2} M; YT 1 mg/mL: $p = 0.036$ (**) for $CaCl_2$ 10^{-3} M and $p = 0.0011$ (**) for $CaCl_2$ 10^{-2} M. (b) OT 0.3 mg/mL: $p = 0.0250$ (**) for $CaCl_2$ 10^{-2} M; OT 1 mg/mL: $p = 0.0076$ (**) for $CaCl_2$ 10^{-3} M and $p = 0.0002$ (***) for $CaCl_2$ 10^{-2} M. (c) Effect of pre-incubation in Ca^{2+}-free krebs with solvent (control, C), YT, and OT extracts (0.3 and 1 mg/mL) on tension induced by PE (10^{-6} M) in endothelium-intact rat aortic rings. Data are present as mean ± SEM ($n = 6$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. control (solvent alone). Statistical analysis: paired two-tailed student's t-test. YT 0.3 mg/mL: $p = 0.0110$ (*), YT 1 mg/mL: $p = 0.0022$ (**); OT 0.3 mg/mL: $p = 0.0026$ (**); and OT 1 mg/mL: $p = 0.0130$ (*).

tested doses (0.3, 1, and 2 mg/mL) compared with the control. Glib significantly reduced the vasorelaxant response of YT extract at the doses of 1 and 2 mg/mL (42.57% and 39.2%, respectively). However, the vasorelaxant effect of YT extract was significantly inhibited by all doses of IbTx (89.8%, 61.7% and 57.3%, respectively), $BaCl_2$ (81.9%, 55.0% and 44.6%, respectively), and 4-AP (82.4%, 62.1% and 38.3%, respectively) (Figure 5(a)).

In the case of OT extract, the relaxing response was not significantly reduced by apamin and TRAM-34, although to a lesser extent than that for the YT extract. At a dose of 0.3 mg/mL, the vasorelaxant response of OT extract was significantly reduced only by $BaCl_2$. However, at doses of 1 and 2 mg/mL, the relaxing response of the OT extract was significantly reduced by the same potassium channel inhibitors as with the YT extracts: IbTx (42.3% and 32.7%, respectively), Glib (35.5% and 35.8%, respectively), $BaCl_2$ (61.0% and 51.3%, respectively) and 4-AP (52.7% and 23.9%, respectively) (Figure 5(b)). The potassium channels inhibitors did not show any effect on basal muscle tone per se.

These results suggest that the vasorelaxant effect of *T. erecta* is mainly mediated by potassium channels located in the smooth muscle, specifically BK_{Ca}, K_{ATP}, K_{IR}, and K_V.

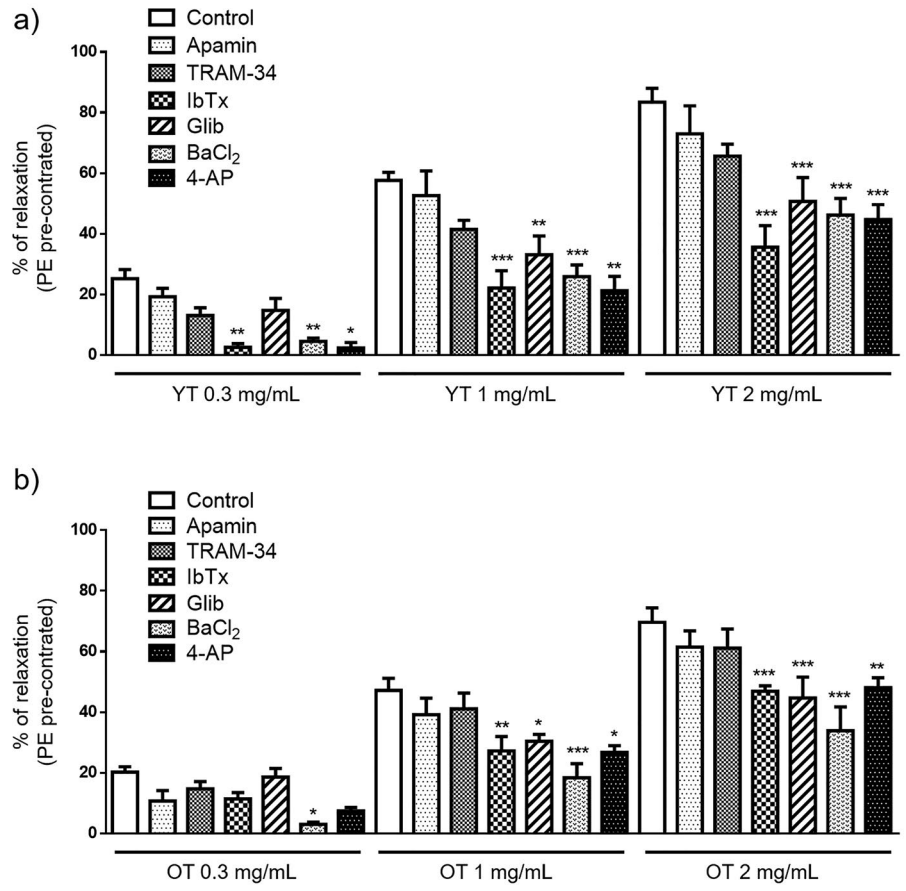

Figure 5. Effect of pre-incubation with potassium channel inhibitors apamin (AP, 10^{-6} M), TRAM-34 (10^{-6} M), iberiotoxin (IbTX, 3×10^{-8} M), glibenclamide (Glib, 10^{-5} M), $BaCl_2$ (3×10^{-5} M), and 4-aminopyridine (4-AP, 10^{-3} M) on the vasorelaxant response to (a) yellow cultivar (YT) and (b) orange cultivar (OT) extracts (0.3, 1, and 2 mg/mL) in isolated endothelium-intact rat aortic rings pre-contracted with PE (10^{-6} M). Data are expressed as mean ± SEM ($n=6$–8). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs. control (extract alone). Statistical analysis: two-way ANOVA followed by dunnett post hoc test. For YT: Interaction: $F=1.054$, $p=0.405$; dose: $F=157.8$, $p\leq0.0001$, inhibitor treatment: $F=24.72$, $p\leq0.0001$. (YT 0.3 mg/mL: $p=0.0031$ (**) for IbTX; $p=0.0054$ (**) for $BaCl_2$; and $p=0.0245$ (*) for 4-AP. YT 1 mg/mL: $p\leq0.0001$ (***) for IbTX; $p=0.0023$ (**) for Glib; $p\leq0.0001$ (***) for $BaCl_2$; and $p=0.0006$ (***) for 4-AP. YT 2 mg/mL: $p\leq0.0001$ (****) for IbTX, Glib, $BaCl_2$; and 4-AP). For OT: Interaction: $F=1.246$, $p=0.2589$; dose: $F=146.6$, $p\leq0.0001$, inhibitor treatment: $F=15.10$, $p\leq0.0001$). (OT 0.3 mg/mL: $p=0.0100$ (*) for $BaCl_2$. OT 1 mg/mL: $p=0.0025$ (**) for IbTX; $p=0.0238$ (*) for Glib; $p\leq0.0001$ (***) for $BaCl_2$; and $p=0.0019$ (**) for 4-AP. OT 2 mg/mL: $p\leq0.0001$ (***) for IbTX; $p=0.0007$ (***) for Glib; $p\leq0.0001$ (***) for $BaCl_2$; and $p=0.0065$ (**) for 4-AP).

Effect of PKA on T. erecta extract-induced vasorelaxation

To investigate whether *T. erecta*-induced vasodilation is mediated by PKA signaling, endothelium-intact aortic rings were pre-incubated with H-89 (2×10^{-7} M), a PKA inhibitor. After pre-incubation, the vasorelaxant responses of the extracts (0.3, 1, and 2 mg/mL) were assessed in aortic rings pre-contracted with PE (control). Pretreatment with H-89 significantly reduced the vasorelaxation response compared to the control, with reductions of 28.9% and 21.3% for YT extract at 1 and 2 mg/mL, respectively, and 17.2% for OT extract at 2 mg/mL (Figure 6). These results suggest that PKA could play a role in the vasorelaxant response induced by *T. erecta* extracts.

Effect of myosin light chain phosphatase on the response to T. erecta extracts

The possible effect of myosin light chain phosphatase (MLCP) on the relaxant response evoked by *T. erecta* extracts was investigated. Pre-incubation of endothelium-intact aortic rings with okadaic acid (10^{-6} M), a potent inhibitor of MLCP, did not alter the vasorelaxant effect of YT and OT extracts (Table 2).

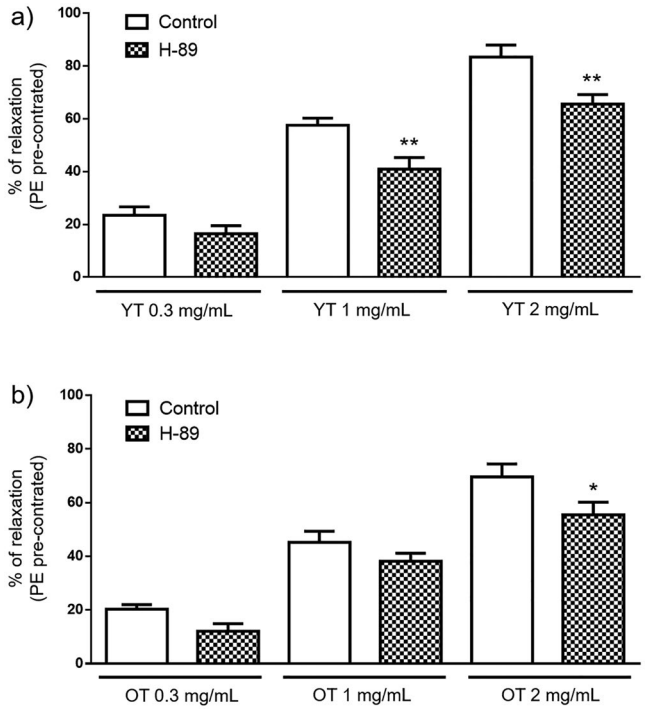

Figure 6. Relaxant effects of (a) yellow cultivar (YT) and (b) orange cultivar (OT) extracts in the absence (control) and presence of H-89 (2×10^{-7} M), a protein kinase a inhibitor, on PE-induced contractions in isolated endothelium-intact rat aortic rings. Data points are presented as mean ± SEM ($n=6$–8). *$p<0.05$ and **$p<0.01$ vs. control (extract alone). Statistical analysis: paired two-tailed student's t-test. (YT 1 mg/mL: $p=0.0058$ (**); YT 2 mg/mL: $p=0.0088$ (**); OT 2 mg/mL: $p=0.0496$ (*).

Table 2. Percentage of relaxation induced by yellow (YT) and orange (OT) cultivar extracts in the absence and presence of okadaic acid (OAc, 10^{-6} M) on phenylephrine-induced contractions in endothelium-intact aortic rings.

mg/mL	YT	OAc + YT	OT	OAc + OT
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.01	0.4 ± 0.9	1.1 ± 0.2	1.2 ± 0.5	0.6 ± 0.2
0.03	4.0 ± 1.9	3.2 ± 0.5	5.8 ± 0.8	3.2 ± 1.1
0.1	12.0 ± 2.1	9.0 ± 1.6	12.5 ± 1.2	8.3 ± 2.9
0.3	25.17 ± 3.0	19.8 ± 1.4	20.3 ± 1.7	19.5 ± 6.0
0.5	39.8 ± 3.7	32.2 ± 4.4	31.7 ± 2.7	31.8 ± 9.3
1	57.6 ± 2.6	55.7 ± 7.1	47.2 ± 3.9	47.5 ± 9.6
2	83.4 ± 4.2	77.3 ± 6.4	69.7 ± 4.1	62.5 ± 6.9

Data points are presented as mean ± SEM ($n=6$–12). Statistical analysis: paired two-tailed Student's t-test.

In vivo assays

Pharyngeal pumping

The pharyngeal contraction of *C. elegans* exposed to the different conditions is shown in Figure 7. No differences in pharyngeal pumping were observed between obese worms (5% glucose) and control worms (NGM). However, worms exposed to 250 µg/mL of *T. erecta* extracts with 5% glucose showed a reduction in the rate of pharyngeal pumping compared to the obese worms (5% glucose), approximately 10% and 11% on days 2 and 6, respectively.

Discussion

T. erecta, also known as Mexican or African marigold, is an ornamental and medicinal plant widely used for both decorative and therapeutic purposes, as well as for the extraction of lutein due to its high concentration of carotenoids. One of its traditional uses in medicine has been for the treatment of hypertension (Michel et al. 2020). However, there are no studies supporting its use as an

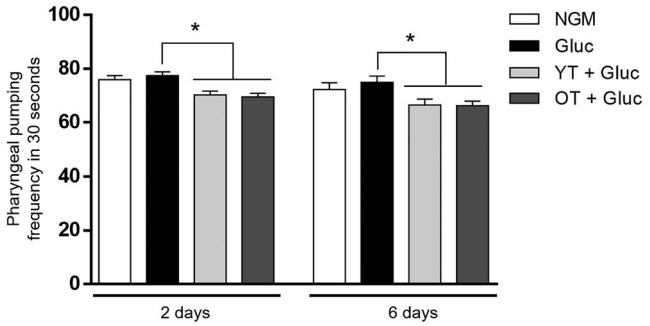

Figure 7. Pharyngeal pumping rate per 30 s in *C. elegans* after exposure to different conditions: NGM (nematode growth medium), gluc (5% glucose), YT + gluc (yellow cultivar extract + glucose), and OT + gluc (orange cultivar extract + glucose), measured at 2 and 6 days. Results are expressed as mean ± SEM ($n = 30–45$). *$p < 0.05$ vs. gluc. *$p < 0.05$ vs. gluc. Statistical analysis: ANOVA test followed by dunnett post hoc test. (2 days: $p = 0.0288$ (*) for YT + gluc; and $p = 0.0172$ (**) for OT + gluc. 6 days: $p = 0.0313$ for YT + gluc; and $p = 0,0265$ for OT + gluc).

antihypertensive agent. Therefore, this study evaluates the effect of two ethanolic extracts of *T. erecta* on vascular tone and their potential as vasorelaxant agents.

First, *T. erecta* extracts showed a concentration-dependent vasorelaxant response in endothelium-intact aortic rings pre-contracted with PE. This vasorelaxant response was also observed in endothelium-denuded rings pre-incubated with PE, when the endothelium was mechanically removed. However, the relaxing response obtained by the extract at low doses was significantly lower than in the presence of endothelium, while at high doses, the same vasorelaxant response was observed. These results suggest that the absence of endothelium partially affects the vasorelaxant activity of *T. erecta* extracts, but vasorelaxant effects are still observed, indicating the involvement of endothelium-independent pathways in the vasorelaxant action.

To further investigate the participation of the endothelium in the relaxing effect of *T. erecta*, endothelium-derived relaxing factors, such as nitric oxide (NO) and prostacyclin I2 (PGI2), were evaluated. The formation of NO in endothelial cells, a potent vasodilator gas, requires the enzyme nitric oxide synthases (NOS), with endothelial NOS (eNOS) being the main isoform involved in the regulation of vascular activity. NO diffuses into vascular smooth muscle cells (VSMCs) and activates soluble guanylate cyclase (sGC), which in turn activates protein kinase G (PKG), leading to a decrease in intracellular calcium and vasodilation (Godo and Shimokawa 2017; Konukoglu and Uzun 2017). Our results show that pre-incubation of endothelium-intact aortic rings with L-NAME (NO-synthase blocker) and ODQ (sGC inhibitor) attenuated the relaxing response evoked by low doses of *T. erecta* but did not affect the relaxation induced by higher doses. These results indicate that the relaxant effect of *T. erecta* extracts is partially mediated by NO production, which relaxes VSMCs through activation of sGC and the subsequent cGMP production.

Moreover, our study demonstrated that pre-incubation of endothelium-intact rings with atropine, a competitive antagonist of muscarinic receptors, decreased the vasorelaxant response of *T. erecta* extracts at 0.1 and 0.3 mg/mL. Acetylcholine binds to M_3 muscarinic receptors on endothelial cells and stimulates eNOS, which increases NO synthesis and consequently induces relaxation (Walch et al. 2001). These data suggest that *T. erecta* extracts, in part, exert their relaxing action through an endothelium-dependent mechanism mediated by NO, but not exclusively, indicating that the *T. erecta* extracts primarily act directly on vascular smooth muscle.

An *in vivo* study demonstrated diuretic and natriuretic effects following oral administration of very low doses of a hydroethanolic extract of *T. erecta* in both normotensive and hypertensive rats. This diuretic potential was evidenced by a significant increase in urine volume and Na^+ excretion (Zanovello et al. 2021). Consistent with our results, the study observed that oral pretreatment with atropine decreased the diuresis induced by the extract while maintaining its natriuretic effect. These results suggest an endothelium-dependent vasorelaxant effect of *T. erecta* on the renal vessels. Furthermore, this work does not rule out the possibility of direct effects of the compounds present

in the extract on the cation transporters in the nephron tubule, which could modify the diuretic effect (Zanovello et al. 2021).

PGI2 is a vasodilator molecule produced by endothelial cells through the cyclooxygenase enzyme. Unlike NO, PGI2 binds to prostacyclin receptors on the membranes of VSMC and elevates cytosolic cAMP levels, which promotes the activation of protein kinase A (PKA), favoring membrane hyperpolarization, reducing intracellular Ca^{2+} and inducing relaxation in vascular smooth muscles (Roberts and Dart 2014). Pre-incubation with indomethacin (a cyclooxygenase blocker) in endothelium-intact aortic rings did not modify the relaxing response of *T. erecta* to PE pre-contraction, indicating that the vasorelaxant effect may not be induced by prostaglandins or prostacyclin.

In a study conducted with another species of *Tagetes*, *T. lucida*, its ethanolic extract showed an antihypertensive effect in spontaneously hypertensive rats and a vasorelaxant effect on isolated aortic rings. Similar to our results, the vasorelaxant effect in endothelium-denuded rings was significantly reduced at low doses compared to the endothelium-intact rings, but not at high doses. Furthermore, the vasorelaxant effect was reduced by L-NAME or ODQ, but not by indomethacin, indicating that the effect was partially endothelium-dependent through NO/cGMP pathway (Estrada-Soto et al. 2021).

This finding is particularly interesting for the treatment of pathologies with endothelial dysfunction, such as hypertension, where the production and bioavailability of NO are reduced and/or its cellular functions are altered (Godo and Shimokawa 2017; Konukoglu and Uzun 2017; Gallo et al. 2021).

In our study, in addition to attenuating the contractile response in endothelium-intact aortic rings pre-contracted with PE, this response was also observed with other vasoconstrictor agents such as 5-HT and K^+. PE induces vasoconstriction by binding to α-1 adrenergic receptors on VSMC, triggering intracellular events that result in the release of calcium from the sarcoplasmic reticulum (SR) due to an increase in IP_3 levels and the influx of extracellular Ca^{2+} through receptor-operated and store-operated Ca^{2+} channels (ROCC and SOCC). The increase in intracellular Ca^{2+} concentration activates myosin light chain kinase (MLCK) through the calcium-calmodulin complex, allowing the myosin-actin interaction and contraction of vascular smooth muscle (Touyz et al. 2018). Similarly, 5-HT acts on the vasculature *via* the $5\text{-}HT_{2A}$ receptor, producing the release of intracellular Ca^{2+} by increasing the IP_3 levels and facilitating the capacitive entry of Ca^{2+} (dos Santos Oliveira and Silva 2011; Sung et al. 2013; Guner et al. 2022). It also induces vasoconstriction by inhibiting the K_V channel, although the involvement of other potassium channels as K_{ATP}, K_{IR}, or BK_{Ca}, in this response remains controversial (Sung et al. 2013). On the other hand, vasoconstriction induced by a K^+-rich medium occurs due to the depolarization of the VSMC membrane, which activates voltage-dependent calcium channels (VDCC), causing an influx of extracellular calcium. This calcium increase leads to the phosphorylation of MLC and the contraction of VSMC (Dogan et al. 2019). The reduced contractile response to these vasoconstrictors in the presence of the *T. erecta* extracts suggests that the extracts may exert their effects by inhibiting the PLC-IP_3-DAG-PKC pathway, reducing intracellular calcium levels, and/or promoting the opening of potassium channels.

The vasorelaxant effects of the extracts varied depending on the contractile agent, following the order: PE ≫ 5-HT ⋙ KCl. The minor response of the extract in a K^+-rich medium could suggest that *T. erecta* extracts may play a role in the opening of K^+ channels in VSMCs. Opening of potassium channels in VSMCs causes potassium efflux, leading to membrane hyperpolarization, closure of VDCCs, reduced calcium influx, and subsequent vasodilation (Dogan et al. 2019). On the other hand, if the potassium concentration in the extracellular medium is very high, potassium will not be able to exit the cell, and therefore, the cell will not be able to relax.

Several studies suggest that increased activation of the PLC-IP_3-DAG-PKC pathway and elevated intracellular calcium levels contribute to impaired vascular regulation, promoting contractility and leading to increased blood pressure, which results in hypertension. Likewise, the functional down-regulation of potassium channels may also be linked with an increase in intracellular calcium (Meier and King 2000; Pintérová et al. 2011; Khalil 2013).

Ca^{2+} plays a very important role in the contraction of smooth muscle and other functions that involve cellular phenotype and function. In the blood vessels, calcium homeostasis is essential for regulating vascular tone by modifying the diameter of the vessel and resistance to blood flow, which

explains its involvement in the pathogenesis of diseases such as hypertension (Touyz et al. 2018; Gallo et al. 2021).

In relation to calcium, in this study, pretreatment with *T. erecta* extracts reduced the vasoconstriction induced by PE and Ang II in a calcium medium. The effect of the higher concentration of the extracts was similar to or greater than that produced by verapamil. Ang II is a key vasoconstrictor in the renin-angiotensin-aldosterone system, regulating blood pressure by binding to AT-1 receptors. This binding increases peripheral resistance and blood pressure through the release of Ca^{2+} stored in the SR and the subsequent entry of extracellular Ca^{2+} through VDCCs (Kanaide et al. 2003; Te Riet et al. 2015; Touyz et al. 2018; Mirabito Colafella et al. 2019; Gallo et al. 2021). Also, a recent study demonstrated that the vasoconstrictive effect of Ang II is, in part, due to its ability to stimulate endocytosis and degradation of BK_{Ca} channels (Yin et al. 2019). In our study, we observed that pretreatment with *T. erecta* extract also reduced vasoconstriction induced by the addition of $CaCl_2$ and PE in a Ca^{2+}-free medium enriched with potassium. This result demonstrates that *T. erecta* blocks both the entry of extracellular calcium and the release of calcium from the SR in VSMCs. These results highlight that *T. erecta* could act as an antagonist of VDCCs channels and interact with the IP_3 signaling pathway in the vasodilator response.

This result aligns with the effect of the aqueous extract of *T. erecta* in the guinea pig ileum, where we demonstrated that the spasmolytic effect of *T. erecta* was similar to that produced by verapamil, although with less intensity. The authors suggest that this effect could, in part, be due to its impact on L-type Ca^{2+} voltage-dependent channels (Ventura-Martínez et al. 2018). Additionally, the ethanolic extract of *T. lucida* blocked L-type calcium channels in aortic rings, exhibiting the same mechanism of action as the *T. erecta* extracts (Estrada-Soto et al. 2021).

On the other hand, alterations in potassium channels can cause membrane depolarization, triggering vasoconstriction and hypertension. In our study, pre-incubation of endothelium-intact aortic rings with IbTx, an antagonist of big-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), Glib, an inhibitor of ATP-sensitive K^+ channels (K_{ATP}), $BaCl_2$, an inhibitor of inward rectifier K^+ channels (K_{IR}) and 4-AP, an inhibitor of the voltage dependent K^+ channel (Kv), significantly decreased the vasorelaxant response to the *T. erecta* extract. However, TRAM-34 and apamin, selective inhibitors of endothelial Ca^{2+}-activated K^+ channels of intermediate (IK_{Ca}) and small conductance (SK_{Ca}), respectively, also reduced the response to *T. erecta* extract, but this reduction was not statistically significant. These results suggest that the vasorelaxant effect of *T. erecta* extract (Figure 8) involves the opening of at least four potassium channels in arterial smooth muscle cell membranes: BK_{Ca}, K_{ATP}, K_{IR} and Kv. Meanwhile, the IK_{Ca} and SK_{Ca} channels present in vascular endothelial cells appear to play a lesser role in the vasorelaxation induced by the extracts. Overall, these results would indicate that the main vasorelaxant effect of *T. erecta* is independent of the endothelium.

K^+ channel activity plays a crucial role in regulating vessel diameter and vascular tone by determining the resting membrane potential in VSMCs. Changes in the structure and function of K^+ channels have been associated with vascular diseases such as hypertension, atherosclerosis, and diabetes mellitus, in which increased vascular tone is associated with alterations in the resting potential of VSMCs. These changes can lead to abnormal vascular tone and heightened sensitivity to vasoconstrictive substances. As a result, K^+ channels have become important therapeutic targets for the treatment of vascular diseases (Pintérová et al. 2011; Dogan et al. 2019).

These K^+ channels can be activated directly by vasodilator agents or through the activation of protein kinases. An increase in intracellular cAMP levels activates PKA in VSMCs, inducing membrane hyperpolarization. This hyperpolarization increases the probability of K^+ channel opening, leading to vasorelaxation (Pintérová et al. 2011; Valero et al. 2011; Jackson 2017; Dogan et al. 2019).

Our results indicated that *T. erecta*-induced relaxation in aortic rings was inhibited by H-89, a protein kinase A blocker, suggesting the involvement of PKA in this relaxation response. In VSMCs, the increase in PKA can occur through several signaling pathways, in addition to prostacyclin receptors, such as through Gs protein-coupled receptors like β-adrenergic or adenosine receptors, or through ATP-sensitive receptors, such as purinergic receptors (Kyle Hogarth et al. 2004; Nash et al. 2018; Moser et al. 2021). Therefore, the relaxing effect of *T. erecta* extract could be attributed to an increase

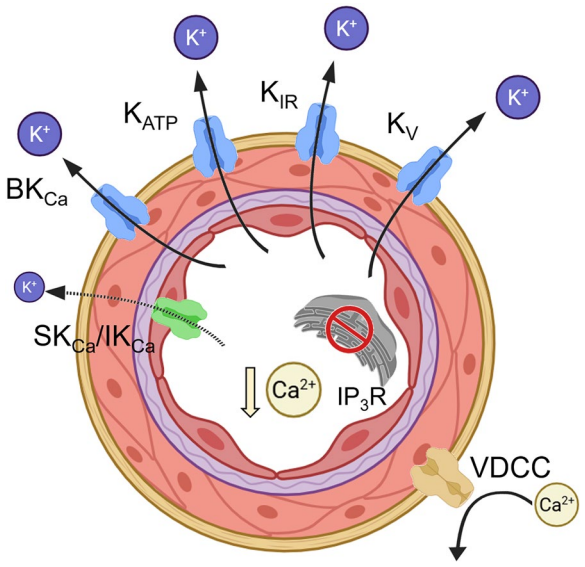

Figure 8. Diagram of the vasorelaxant activity of *T. erecta* extracts on aortic rings. BK_{Ca}: big-conductance Ca^{2+}-activated K^+ channel; IK_{Ca}: intermediate-conductance Ca^{2+}-activated K^+ channel; SK_{Ca}: small-conductance Ca^{2+}-activated K^+ channel; K_{ATP}: ATP-sensitive K^+ channel; K_{IR}: inward-rectifier K^+ channel; K_V: voltage-dependent K^+ channel; IP_3R: inositol 1,4,5-trisphosphate receptor; VDCC: voltage-dependent calcium channels.

in intracellular PKA levels, which leads to the opening of K^+ channels (BK_{Ca}, K_{ATP}, K_{IR}, and K_V), leading to membrane hyperpolarization and a vasorelaxant effect (Jackson 2017).

Finally, inhibition of MLCK did not modify the response to the extract, suggesting that the *T. erecta* extract does not act directly on MLCK.

In the *in vivo* model, obese worms treated with *T. erecta* extracts showed a reduced pharyngeal pumping rate compared to untreated obese worms. These results are similar to those obtained in a recent study, in which ethanolic extracts of two *T. erecta* cultivars obtained by soxhlet extraction produced similar results, but only at a higher dose (500 µg/mL) (Núñez et al. 2023). The *C. elegans* pharynx shares molecular and structural similarities with the human heart and has been used as an arrhythmic model to study the effects of new drugs and compounds with potential applications in cardiovascular diseases (Srinivasan et al. 2023). Pharyngeal pumping consists of coordinated cycles of relaxation and contraction of the pharyngeal muscle. The action potential produced to generate the pumping movement is achieved through the sequential action of five ion channels: a nicotinic acetylcholine receptor, a low- and high-threshold voltage-dependent calcium channel, a glutamate-dependent chloride channel, and voltage-dependent potassium channels. The effects of antiarrhythmic drugs in *C. elegans* are influenced by several mechanisms, primarily involving calcium signaling and ion channel modulation (Avery and You 2012; Jiang et al. 2018). These results suggest that *T. erecta* extracts could reduce heart rate in mammals by blocking the increase in cytosolic calcium and/or by opening Kv channels, thereby lowering blood pressure. Nevertheless, although the *C. elegans* pharyngeal pumping assay provides a useful functional analogue of rhythmic contractile activity, it should not be interpreted as a direct surrogate for mammalian cardiac function. Further *in vivo* studies in mammalian models are required to evaluate the effects of *T. erecta* extracts on cardiac output and arterial pressure.

The vasorelaxant effect and decrease in pharyngeal pumping by *T. erecta* extracts may be attributed to their phytochemical composition. Furthermore, differences in the vasorelaxant response between the two extracts have been observed, probably due to variations in their composition (Núñez et al. 2025). Plant-derived compounds have been shown to improve cardiovascular health and manage hypertension by releasing endothelium-derived relaxing factors such as NO and PGI2, opening K^+ channels, and inhibiting intracellular calcium (Ajay et al. 2003; Chiwororo and Ojewole 2010; Luna-Vázquez et al. 2013; Grosso et al. 2022). In the study of *T. lucida*, some compounds showed relaxing effects, which were partially endothelium-dependent, while other compounds produced the

same effect regardless of the presence of the endothelium, as its removal did not modify their relaxing response (Estrada-Soto et al. 2021).

Similarly, the vasorelaxant effect of another plant belonging to the Asteraceae family, specifically *Helichrysum stoechas* ('*The Potential Role of Everlasting Flower (Helichrysum stoechas Moench) as an Antihypertensive Agent: Vasorelaxant Effects in the Rat Aorta*'; PI66/17; Valero et al. 2022), was studied simultaneously. Although this extract also exhibited a potent relaxing effect on vascular smooth muscle, *Tagetes erecta* extracts stand out as vasorelaxants due to their primarily endothelium-independent mechanisms of action. This makes *T. erecta* particularly interesting for individuals with hypertension associated with endothelial dysfunction. These differences could be attributed to variations in their phytochemical composition.

Polyphenols such as caffeic acid, ellagic acid, kaempferol, quercetin, and luteolin present in our extract exert vasorelaxant effects either directly on VSMCs or indirectly through potassium channels (Ajay et al. 2003; Chiwororo and Ojewole 2010; Luna-Vázquez et al. 2013; Grosso et al. 2022). Flavonoids primarily induce vasodilation by inhibiting PKC, but other mechanisms, such as the inhibition of kinases, phosphodiesterases, and the blockade of Ca^{2+} entry, may also contribute to this effect (Álvarez Castro and Cambeiro 2003). For example, quercetin and kaempferol exert vasorelaxant activity that is partly endothelium-dependent, mediated through pathways involving NO-cGMP-PKG and PGI2, as well as endothelium-independent, through mechanisms involving PKC, BK_{Ca} channels, L-type Ca^{2+} channels, and PKA signaling (Duarte et al. 1993; Chiwororo and Ojewole 2010; Suri et al. 2010; Mahobiya et al. 2018).

Pigments such as carotenoids and tocopherols present in our extract have been shown to benefit the cardiovascular system by improving endothelial function and metabolic profile. β-carotene act as hypotensive agents by enhancing the NO system, having antioxidant properties, and inhibiting angiotensin-converting enzyme expression (Wigg et al. 2004; Abbasian et al. 2023; Behzadi et al. 2025). Vitamin E supplementation improves vascular complications, such as diabetes-induced endothelial dysfunction, by directly affecting smooth muscle through PKC inhibition (Wigg et al. 2004).

Conclusions

This is the first study to demonstrate that both cultivars of *T. erecta*, yellow and orange, exhibit vasorelaxant properties in rat aorta, with NO-dependent effects at low concentrations and NO-independent effects at high doses, as well as a reduction in pharyngeal pumping in *C. elegans*. *T. erecta* may act through the NO/sGC pathway, by opening BK_{Ca}, K_{ATP}, K_{IR}, and K_V channels, activating the PKA pathway, and reducing intracellular calcium by inhibiting both Ca^{2+} influx and Ca^{2+} release from the sarcoplasmic reticulum. While *C. elegans* results support the observed vasorelaxant effects, further validation in mammalian models remains necessary.

Considering that *T. erecta* edible flowers are a source of a wide variety of phytochemicals, different mechanisms may be implicated at the smooth muscle level, making them a potentially useful herbal remedy for the prevention of pathologies associated with endothelial dysfunction, such as hypertension, atherosclerosis, and diabetes.

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Ethical approval

No experimental animals were used in this study, as surplus tissue obtained from animals used in another project - identified by the ethics committee with reference number PI66/17 - was used.

Author contributions

CRediT: **Sonia Núñez**: Data curation, Investigation, Writing – original draft; **Victor López:** Funding acquisition, Project administration, Writing – review & editing; **María Pilar Arruebo Loshuertos**: Conceptualization, Methodology, Supervision; **Miguel Ángel Plaza Carrión**: Methodology, Supervision, Validation, Writing – review & editing; **Carlota Gómez-Rincón**: Conceptualization, Methodology, Supervision, Validation; **Marta Sofía Valero**: Conceptualization, Data curation, Investigation, Supervision, Writing – original draft.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

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