Calcium Channel Blockade Mediates the Vasorelaxant Activity of Dichloromethane Extract from Roots of \textit{Oncidium cebolleta} on Isolated Rat Aorta

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Abstract: \textit{Oncidium cebolleta} belongs to the Orchidaceae family, and it is used in preparations in Mexican traditional medicine to treat several diseases. This research aimed to study the vasorelaxant activity of dichloromethane extract from the roots of Oncidium cebolleta (DEROc) on endothelium-intact and -removed rat aorta segments to determine its mode of vasorelaxant action. Several extracts (hexane, dichloromethane, and methanol) obtained from leaves and roots were evaluated on isolated rat aorta rings with and without endothelium to determine their vasorelaxant effect. Except for methanolic extract from leaves, all extracts induced a concentration-dependent vasorelaxant effect on endothelium-intact rat aorta rings pre-contracted with noradrenaline (NA, 0.1 µM). Dichloromethane extract from roots (DEROc) was the most potent and efficient, and the effect was endothelium-independent. Also, DEROc could relax KCl-induced contraction (80 mM) and inhibit the contraction induced by CaCl\textsubscript{2} cumulative concentration on endothelium-denuded rings. However, DEROc was less potent than control nifedipine (L-type calcium channel blocker), but its effectiveness was similar. Constrictions induced by NA and 5-HT [0.001 µM to 0.1 µM] were significantly reduced by DEROc. However, DEROc vasorelaxation was not reduced by TEA (potassium channel blocker, 10 mM) nor by ODQ (soluble guanylyl cyclase inhibitor, 0.1 µM) in endothelium-denuded aortic rings. Our functional results suggest that DEROc induces its relaxant effect by an endothelium-independent mechanism due to a Ca\textsuperscript{2+} channels blockade in rat aortic rings.

Keywords: Orchid; \textit{Oncidium cebolleta}; vasorelaxant; calcium channel blockade.

1. Introduction

In 2016, according to WHO data, 31% of the world’s deaths were from vascular diseases [1] such as endothelial dysfunction, metabolic syndrome, chronic kidney diseases, heart failure, myocardial infarction, stroke, and vascular dementia, which are closely related to hypertension [2,3]. WHO suggests using medicinal plants to improve various chronic diseases with increasing risk worldwide. Thus, the use of orchids in traditional medicine is extensively...
described [4,5]. The Orchidaceae is one of the largest flowering plants family, and this family comprises over twenty thousand members [6]. Oncidium cebolleta (Orchidaceae) is traditionally used to cure foot infections in Mexico [7]; nevertheless, there are no ethnopharmacological statements that indicate its uses for the treatment of hypertension; however, this plant was selected by chemotaxonomic and pharmacological criteria based on previous studies on orchids. Thus, earlier investigations showed that the methanolic extracts from Laelia autumnalis and Laelia anceps possess a significant vasorelaxant activity on contractions induced by noradrenaline in isolated rat aorta rings without depending on the presence of endothelium [8,9]. Another study demonstrated that the extract of Orchis mascula inhibited PHE/K⁺-induced contractions in isolated rabbit aorta. Further, the same extract reduced systolic blood pressure and improved endothelial function in SHR rats [10]. It was recently observed that Dendrobium officinale ultrafine powder improved the vascular endothelial relaxation function and lowered blood pressure [11], decreasing the levels of total cholesterol, triglyceride, low-density lipoprotein cholesterol, glucose, and insulin in blood in metabolic hypertensive rat models [12]. Moreover, the extract of the orchid Dendrobium officinale showed a significant reduction of systolic and mean arterial blood pressures in hypertensive rats [13]. Overall, this evidence suggests that orchids will be used as alternative drugs and will be considered potential therapies for cardiovascular and cerebrovascular diseases, such as hypertension. Therefore, this research aimed to study the vasorelaxant effect of the dichloromethane extract of Oncidium cebolleta and characterize its possible mode of vasorelaxant action.

2. Materials and Methods

2.1. Chemicals and drugs.

Carbamoylcholine chloride ≥98% (carbachol), noradrenaline HCl (NA), 5-hydroxytryptamine creatinine sulfate (5-HT), nifedipine, 1-H-[1,2,4]-oxadiazolo-[4,3a]-quinoxalin-1-one (ODQ), and tetraethylammonium (TEA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Additional analytical grade reagents were obtained from local suppliers. Regarding in vitro assays, the extracts were dissolved in distilled water dimethyl sulfoxide (DMSO, 1% v/v). At the same time, the remaining reagents were sonicated after being dissolved in distilled water before being added to assay models. DMSO showed no effect on the basal tension of aortic rings.

2.2. Plant collection and extracts preparation.

The O. cebolleta species was harvested from Tres Marías, Morelos, Mexico in June 2007. The identification of the plant was carried out by Dr. Patricia Catillo-España. A voucher specimen (# 10889) was deposited at the HUMO-Herbarium at the “Centro de Estudios Ambientale e Investigación “Sierra de Huautla” (CEAMISH)” of the Morelos State University. The plant material was separated into roots and leaves. Then, the dried plant material was pulverized (leaves, 61.3 g and roots, 44 g), and crude extracts were prepared by successive maceration with hexane, dichloromethane, and methanol, three times for 72 h at room temperature. After filtration, extracts were concentrated under vacuum pressure using a rotary evaporator (Buchi® Heating Bath B-490) at 40° C.
2.3. Animals.

Male Wistar rats weighing 250-350 g were used. Animals were exposed to a normal light cycle and were maintained at room temperature with water and chow ad libitum. All animals were cared for following our Federal Regulations for Animal Experimentation and Care (SAGARPA, NOM-062-ZO-1999, Mexico) and approved by the Institutional Animal Care and Use Committee (Protocol 1368, F.E.S. Iztacala) based on US National Institute of Health publication (No. 85-23, revised 1985). All experiments were carried out using six animals per group. Wistar rats were provided by F.E.S. Iztacala, from Universidad Nacional Autónoma de México.

2.4. Preparation of rat aortic rings.

The protocol was conducted according to Arias-Duran et al. [14]. In brief, rats were anesthetized in an ether-saturated chamber, and then the thoracic aorta was dissected and placed in Krebs-Henseleit buffer at room temperature. Aorta was cut in rings (4-5 mm in length), cleaned from surrounding connective and fat tissues (in some aortic rings, the intima layer was scraped off with a rugous device), and hooked at the bottom of the chamber and to a Grass-FT03 isometric force transducer (Astromed®, West Warwick, RI, USA), then bathed in Krebs solution [14]. Isometric tension was determined by coupling Grass-FT03 with an MP100 analyzer (Biopac® Instruments, Santa Barbara, CA, USA). The following experiments were conducted to determine the vasorelaxant effect of extracts and establish the mode of action of the most active.

2.5. Effect of extracts on the contraction induced by NA (0.1 µM).

After stabilizing (30 min), the aorta segments were stimulated with noradrenaline (NA, 0.1 µM) for 10 min, and washed out to remove the contractile agent; this procedure was developed every 30 min for 2 h. In the last stimulation, carbachol (1 µM) was added to corroborate the presence or absence of endothelium. Once the plateau was attained, the extracts were added to the bath in quarter-log cumulative concentrations (0.30 µg/mL to 100 µg/mL). Finally, the relaxant effect was determined by comparing maximum vascular contraction before and after adding samples. As positive controls, in the presence or absence of endothelium, carbachol and nifedipine were used, respectively.

2.6. Inhibition produced by DEROc on the contraction provoked by NA and 5-HT.

The aorta segments with the removed endothelium were treated with 15, 30, and 75 µg/mL for 15 min; then NA and 5-HT were separately added at different concentrations (0.001 µM to 0.1 µM). Finally, the contractile effect induced by NA and 5-HT were compared in the absence (control group) and presence of DEROc.

2.7. Relaxant effect of DEROc on the contraction produced by KCl (80 mM).

To establish the possible calcium channels blockade as possible vasorelaxant mechanism induced by DEROc, on aortic rings without endothelium, modifications to the described experimental design were used. For this protocol [14], the aorta segments with the removed endothelium were contracted with KCl (80 mM in the physiological medium). Once the plateau
was attained, concentration-response curves of DEROc-induced relaxation (3.03 µg/mL to 1000 µg/mL) were obtained.

2.8. inhibition produced by DEROc on the contraction provoked by cumulative concentrations of CaCl\(_2\).

To corroborate whether the blockade of the influx of extracellular calcium is involved in the relaxing effect of DEROc, the experiments were carried out in a Ca\(^{2+}\)-free Krebs solution. Aorta segments with the removed endothelium were rinsed with Ca\(^{2+}\)-free solution for 20 min, then cleaned with Ca\(^{2+}\)-free solution containing KCl (80 mM). After all, concentration-response curves for CaCl\(_2\) (0.060 mM to 20 mM) were constructed in the absence of the extract (control group) or after 15 min incubation with it (15 and 75 µg/mL). Finally, the contractile effect induced by CaCl\(_2\) was compared in the absence (control group) and presence of the extract.

2.9. Role of K\(^+\) channel in DEROc-induced relaxation.

Arterial segments without endothelium were treated with tetraethylammonium [a K\(^+\) channel blocker (TEA), 10 mM], 15 min before NA-induced contraction (0.1 µM), and then DEROc was added cumulatively (0.30 µg/mL to 100 µg/mL). The vasorelaxant effect was compared in the absence (control group) and presence of TEA.

2.10. Role of sGC in DEROc-induced relaxation.

Aorta fragments without endothelium were treated with ODQ (0.1 µM), a specific inhibitor of soluble guanylyl cyclase, for 15 min. Then, tissues were contracted with NA (0.1 µM), and once the plateau was attained, relaxant curves of DEROc (0.30 µg/mL to 100 µg/mL) were obtained with increasing cumulative concentrations of the extract. Finally, the relaxant effect induced by DEROc was compared in the absence (control group) and presence of ODQ.

2.11. Data analysis.

Data were expressed as means ± standard error of the mean (S.E.M.). Concentration-response curves were plotted, and the obtained experimental data were adjusted by the nonlinear curves fitting program Origin\(^{®}\) 8.0. Statistical analysis was conducted using one-way ANOVA (p < 0.05), followed by Bonferroni post hoc test by program SigmaStat\(^{®}\) 3.0.

3. Results and Discussion

3.1. Vasorelaxant effect of the extracts on aortic rings contracted by NA (0.1 µM).

Hexane, dichloromethane and methanolic extracts from leaves (HELOc, DELOc, and MELOc, respectively), and roots (HEROc, DEROc, and MEROc, respectively) of Oncidium cebolleta were obtained (Table 1), and then evaluated on NA (0.1 µM) contracted aortic rings (Figure 1).

| Table 1. The yield of the extracts obtained from Oncidium cebolleta. |
|---------------------------------------------------------------|
| **Leaves yield** | **Roots yield** |
| (% | (g) | (%) | (g) |
| Hexane extract | 0.91 | 0.55 | 0.72 | 0.31 |
| Dichloromethane extract | 0.73 | 0.44 | 2.47 | 1.20 |
| Methanol extract | 3.21 | 1.96 | 4.34 | 1.90 |
Figure 1. Concentration-response curves of the relaxant effect induced by extracts from *Oncidium cebolleta*, on isolated rat aortic rings contracted with NA in the presence of endothelium. A) Roots B) Leaves; Data are expressed as the mean ± S.E.M. of six experiments (p < 0.05).

Table 2 shows that all extracts, except methanolic extract of leaves (*MELOc*), induced a vasorelaxant effect on isolated rat aortic rings contracted with NA (0.1 µM) in the presence of endothelium. Nevertheless, root-derived extracts, such as hexane (*HEROc*) and dichloromethane (*DEROc*), showed a higher vasorelaxant effect in the presence of endothelium of all extracts evaluated.

Table 2. Relaxant effects induced by extracts obtained from *O. cebolleta* on the contraction induced by NA (0.1µM).

|                | E+    | E-    |
|----------------|-------|-------|
|                | EC₅₀  | Eₘ₅₀ | EC₅₀  | Eₘ₅₀ |
| Carbachol      | 0.008 | 74.06 | -     | -    |
| Nitedipine     | -     | -     | 0.167 | 97   |
| HELOc          | 16.41 ± 3.72 | 41.69 ± 3.86 | ND    | ND   |
| HEROc          | 9.85 ±1.84  | 99.37 ± 2.69 | ND    | ND   |
| DELOc          | 30.07 ± 5.33 | 64.20 ± 4.99 | ND    | ND   |
| **DEROc**      | **17.34 ± 2.80** | **93.70 ± 3.69** | **14.92 ± 1.78** | **100.59 ± 2.93** |
| MELOc          | ND    | ND    | ND    | ND   |
| MEROc          | 11.60 ± 2.58 | 72.80 ± 1.63 | ND    | ND   |

Results are presented as mean ± S.E.M., n=6. ND; not determined.

Figure 2. Concentration-response curves of the relaxant effect induced by *DEROc*, on isolated rat aortic rings pre-contracted with NA in the presence and absence of endothelium. Results are expressed as the mean ± S.E.M of six experiments (p < 0.05).
Nevertheless, root-derived extracts, such as hexane (HEROc) and dichloromethane (DEROc), showed a higher vasorelaxant effect in the presence of endothelium of all extracts evaluated. As observed in Figure 2, DEROc was one of the most potent and efficient in a concentration-dependent and endothelium-independent manner.

3.2. Vasorelaxant effect of DEROc on endothelium-denuded aortic rings pre-contracted by a high concentration level of KCl (80 mM).

DEROc induced a significant relaxing effect to the contraction induced by KCl (80 mM), in a concentration-dependent manner with maximal relaxant effect value of (E<sub>max</sub>) 96.6% and IC<sub>50</sub> of 85.5 4 µg/mL. However, DEROc was less potent than nifedipine (E<sub>max</sub> 98.8% and IC<sub>50</sub> 0.005 µg/mL), but its effectiveness was similar to this control (Figure 3).

Figure 3. Concentration-response curves of the relaxant effect induced by DEROc and nifedipine on isolated endothelium-denuded rat aortic rings contracted with KCl (80 mM). Results are expressed as the mean ± S.E.M of six experiments (p < 0.05).

3.3. Vasorelaxant effect of DEROc on endothelium-denuded aortic rings contracted with NA, 5-HT (0.001 µM to 0.1 µM) and CaCl<sub>2</sub> (0.06 mM to 20 mM).

Preincubation of 15, 30, and 75 µg/mL of DEROc inhibited the concentration-response induced contraction of aortic rings with NA and 5-HT, in a concentration-dependent manner and in a similar way (Figure 4A and 4B, respectively).

Figure 4. Inhibitory effect of DEROc on the contraction induced by (A) NA and (B) 5-HT, in endothelium-denuded aortic rings. Results are presented as mean ± S.E.M. n= 6, (p < 0.05).
However, 75 µg/mL of DER0c induced a maximal relaxant effect of almost 60% for NA ($E_{\text{max}}$ 2.55 g) and 61% for 5-HT ($E_{\text{max}}$ 1.48 g). On the other hand, pre-incubation with 15 and 75 µg/mL of DER0c decreased significantly (29%, $E_{\text{max}}$ 2.47 g, and 89%, $E_{\text{max}}$ 0.41g, respectively) the maximal effect induced by CaCl$_2$ ($E_{\text{max}}$ 3.46 g). Nevertheless, 75 µg/mL of DER0c completely decreased the concentration-response contraction by CaCl$_2$ (Figure 5).

**Figure 5.** Inhibitory effect of DER0c on the cumulative-contraction curves dependent on extracellular Ca$^{2+}$ influx in Ca$^{2+}$-free solution. Data are expressed as the mean ± S.E.M. of six experiments (p < 0.05).

3.4. Role of K$^+$ channel in DER0c relaxation.

The K$^+$ channel blocker TEA (10 mM) did not inhibit significantly ($E_{\text{max}}$ 86.46%) DER0c induced relaxation ($E_{\text{max}}$ 100.59%) in endothelium-denuded rings pre-contracted by NA (0.1 µM) (Figure 6).

**Figure 6.** Effects of TEA (10 mM) treatment on DER0c-induced relaxation in endothelium-denuded aortic rings, pre-contracted by NA (0.1 µM). Results are presented as mean ± S.E.M., n= 6, (p< 0.05).

3.5. Role of sGC in DER0c induced relaxation.

ODQ (0.1 µM) did not inhibit the concentration-response relaxation by DER0c (0.30 µg/mL to 100 µg/mL) in endothelium-denuded aortic rings (Figure 7).
Figure 7. Effects of ODQ (1 µM) treatment on DEROc-induced relaxation in endothelium-denuded aortic rings, pre-contracted by NA (0.1 µM). Results are presented as mean ± S.E.M., n= 6, (p< 0.05).

3.6. Discussion.

The present work shows evidence of the significant vasorelaxant effect of the hexane (HEROc), dichloromethane (DEROc), and methanolic (MEROc) extracts from roots of Oncidium cebolleta in a concentration-dependent and endothelium-independent manners. Nevertheless, DEROc was one of the most potent and efficient extracts evaluated on endothelium-denuded aortic rings, possibly due to the increased presence of stilbenoids, which is attributed to the vasorelaxant effect. Accordingly, these results may be associated with the relaxant effect showed from several orchid species where stilbenoids were found, such as Maxilaria densa [15], Scaphyglossis livida [16], and Laelia autumnalis [9], among others. On the other hand, it is important to mention that in another study, five stilbenoids compounds (phenanthrenes) were isolated from Oncidium cebolleta [17], one of them, Nudol was also isolated from Maxillaria densa [15].

Thus, the significant vasorelaxant effect of DEROc, in an endothelium-independent manner, suggests that endothelial vasodilator factors are not involved in this effect. Rather it acts on mechanisms regulating the contraction and relaxation of the smooth muscle cells, such as antagonism of adrenergic receptors, calcium channel blockade, augment of intracellular cGMP concentration, and activation of potassium channels. Therefore, we found that DEROc could inhibit the vasoconstriction induced by NA and 5-HT. In this context, the vasoconstriction induced by NA and 5-HT is regulated by activation of α-adrenergic and serotonergic receptors, which are G-protein-coupled receptors (GPCRs). When GPCR is activated, they further activate phospholipase C (PLC) and lead to the generation of DAG and IP3 (secondary messengers). IP3 releases calcium from the sarcoplasmic reticulum (SR), which activates PKC that phosphorylates myosin light chain kinase and causes contraction [18,19]. Whence, it is suggested that DEROc does not directly inhibit α1-adrenergic nor serotonergic receptors, rather the relaxing effect of DEROc could be induced by a decrease of receptor-operated calcium channels activation (ROCC), which increases calcium, depolarizing the membrane or inducing intracellular changes in second messengers. On the other hand, DEROc was able to inhibit contractility induced by KCl, in a concentration-dependent and endothelium-independent manner; however, it was less potent than nifedipine. The contraction induced by KCl is caused by depolarization of the membrane of smooth muscle cells, which occurs by the activation of voltage-operated calcium channels (VOCC). This mechanism
triggers the influx of Ca2+, ultimately resulting in a contraction. In this context, there are mainly three types of Ca2+ channels in membranes of vascular smooth muscle cells, including VOCC and ROCC, which are regulated by membrane potential-dependent voltage and bind to GPCR, respectively [3]. Then, our results suggest that DEROC might obstruct both VOCC and ROCC Ca2+ channels. It is worth mentioning that nifedipine is an L-type calcium channel blocker, which by blocking calcium entry into the cell, causes vascular smooth muscle relaxation (vasodilation). In this regard, we found that the cumulative concentrations of DEROC induced relaxation on endothelium-denuded rat aortic rings, similarly to nifedipine, suggesting that the extract causes a blockade of Ca2+ influx to smooth muscle cells during the relaxation process. Also, EDROc was able to inhibit CaCl2-induced contraction in endothelium-denuded rings; these results allow us to corroborate the hypothesis that the extract possesses the ability to block Ca2+ influx to the smooth muscle cell, causing vascular relaxation.

On the other hand, there are mainly four types of K+ channels in vascular smooth muscle cells, including Ca2+ activated K+ channels (KCa), which cause efflux of K+ [3]. Once activated, these channels act as negative feedback regulators, hyperpolarizing the cell membrane and blocking the entrance of calcium through VOCC [20]. Whence, K+ channels also change the vascular tone by regulating extracellular Ca2+ influx. Then, we found that the relaxant effect of DEROC was not significantly inhibited by the KCa channel blocker tetraethylammonium (TEA, 10 mM). These findings suggest that opening K+ channels is not involved in the relaxation mechanism of DEROC.

Apart from the mechanism mentioned above of smooth muscle relaxation, there are other relaxant molecules involved, such as endothelial-derived relaxing factor (EDRF), including nitric oxide (NO) and prostacyclin from endothelial cells [21,22]. The NO production, catalyzed by eNOS, diffuses into smooth muscle cells and then enhances cGMP synthesis, which activates dependent protein kinase to cause vasodilation by reducing the intracellular Ca2+ [23–25]. In this regard, we found that soluble guanylyl cyclase inhibitor (ODQ) did not significantly reduce the vasorelaxant effect provoked by DEROC, suggesting that the sGC/cGMP pathway is not involved in DEROC-induced relaxation in endothelium-denuded aorta rings.

Thus, our results indicate that DEROC induced its effect mainly by Ca2+ influx blockade to smooth muscle cells, during the relaxation process, on endothelium-denuded rat aortic rings. These findings are consistent with other reported experimental studies, showing that several orchids exert relaxant effects by blocking L-type Ca2+ channels [9] and interacting with the Ca2+-Calmodulin complex [26]. Furthermore, the presence of stilbenoid compounds was confirmed in different orchid species, presumably responsible for the relaxant effect [8,9,16,26,27].

4. Conclusions

In conclusion, the dichloromethane extract from Oncidium cebolleta showed a vasorelaxant effect by endothelium-independent manner and possibly by Ca2+ influx blockade on endothelium-denuded rat aortic rings. These facts suggest that the extracts deserve detailed studies to be considered as potential therapies for cardiovascular and cerebrovascular diseases, such as hypertension.
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Conflicts of Interest

The authors declare no conflict of interest.

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