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Vasorelaxant Effect of Vitexin, Procyanidin B2 and Isoquercetine on Rat's Aortic Smooth Muscle

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Abstract

Vitexin, procyanidin B_2 and isoquercetine are active compounds found in hawthorn berry fruit. They have several cardioprotective, anti-inflammatory, antioxidant, prooxidant, anti-cancer, antinociceptive, anti-convulsant, and other pharmacological influences; with the possibility of improving memory, anti-diabetic and chemo-preventive properties. The current study looked at the effects of vitexin, procyanidin B_2, and isoquercetine (2x10-6 to 3x10-4 M) on rat aortic smooth muscle cells that had been pre-contracted by phenylephrine (PE). The use of particular inhibitors and blockers to affect induced aortic ring relaxation revealed that the mediated relaxation was dependent on K^+, Ca^{2+} channels, and several EDHFs such as NO, PGI2, and cGMP. The relaxation induced by vitexin was primarily dependent on Kca channel activation, NO, cGMP, and PGI2 pathways activation, but not on K_{ATP}, Kir, or Kv channels. Isoquercetine relaxation was mostly dependent on NO. Procyanidin B_2 caused relaxation that was dependent on the L-type Ca2+ channel. According to these findings Vitexin, procyanidin B_2, and isoquercetine all caused concentration-dependent relaxation in rat aortic smooth muscle. Potassium, Ca^{2+} channels, and EDHFs, particularly NO, were involved in the relaxation caused by these compounds. The activation of PKs, as well as the NO released from the endothelium, which activates Kca channels. and causes relaxation in aortic smooth muscle cells,

Keywords: *vitexin, procyanidin B_2, isoquercetine, aorta, K^+ channel and Ca^{+2} channels blocker.*

Introduction

Vitexin (8-C-b-D-glucopyranosyl-apigenin) is a compound with poor water solubility commonly used to prevent heart diseases (1). It possesses a variety of pharmacological properties, including antioxidant, anti-cancer, antinociceptive, and anti-diabetic effects (2). Its effectiveness has been established in the treatment of a variety of cardiovascular disorders, and it is also utilized as a cardioprotective agent (3,4). It has anti-spasmodic and anti-hypertensive effects (5). Vitexin's hypotensive action is due to its ganglion-blocking capabilities, which include anti-bradykinin, anti-serotonin, and anti-oxidative qualities (6,7). Vitexin also has anti-aggregation, anti-cardiac hypertrophy, and anti-vascular smooth contractility properties (9). Both studies (10, 11) found that vitexin protected myocardium cells against hypoxia.

Procyanidin B_2 is a flavonoid found in nature (12) that exhibits antioxidant, prooxidant, and cardiovascular protective characteristics (14,15), as well as chemopreventive capabilities. Isoquercetine (quercetin3-O-b-D-glucopyranoside) is a flavonoid glucoside that can be found in abundance in medicinal plants, fruits, vegetables, and plant-based foods (16). It also possesses a variety of biological features, including anti-inflammatory actions (17), and

antioxidant activities, which includes lowering ROS levels and lowering lipid peroxidation both in vivo and in vitro (18). The major goals of this study were to look at the in vitro relaxant effects of vitexin, procyanidin B_2, and isoquercetine on rat vascular smooth muscle cells, as well as the potential mechanisms underlying them, to design a superior antihypertensive formulation.

Materials and Methods

Animals

In this investigation, Wister male Albino rats weighing 210–310 g were used, they were reared in Animal House, Department of Biology, College of Science, University of Zakho provided rats, fed on ordinary animal food and water and kept in a well-ventilated area.

Isolation of Aorta and Experimental Protocol

Heparin (1000 units/100 gm) was injected intraperitoneally into the rats and to avoid blood clotting and possibly endothelial damage and left for 30 minutes. Animals were anaesthetized with Ketamine (40 mg/kg) and zyalxine (10 mg/Kg) intraperitoneally. The thoracic aorta was isolated carefully, cleansed of connective and fat tissues, and sliced into 4 mm rings. Each ring was fixed in a 10 ml glass chamber containing Krebs solution by two stainless-steel wires used to connect the ring to the bottom of glass chamber from one end and to the force transducer from the other end under 2 gm tension. The bath solution was heated to 37°C, and the preparation was allowed to equilibrate for 1 hour before adding any drugs. Meanwhile, for one hour, the aortic ring was washed with buffer solution every 15 minutes. The pH of the buffer was maintained at 7.4 by continuous aeration of the physiological solution with a gas mixture consisting of 5% CO_2 and 95% O_2 (19). To study the effect of vitexin, procyanidin B_2 and isoquercetine, the dose response curve was established for each compound by its cumulative addition of different concentrations and the produced responses were recorded. To find out the role of various K^+ and Ca^{2+} channels and endothelium derived hyperpolarizing factors (EDHFs), the tissue was pre-incubated either with desired blocker or inhibitor such as TEA, $BaCl_2$ and 4-AP (1×10^{-3}M), GLIB (1×10^{-5}M), nifedipine ($1x10^{-5}$ M) for 20 minutes or L-NAME (3×10^{-4}M), methylene blue (1x10-5 M) and indomethacin (3x10-5 M) for 30 minutes prior to their precontraction with PE(1mM). In endothelium-denuded experiments, the endothelial layer of the aorta was gently removed by its rubbing with syringe needle covered with a piece of cotton wool. The integrity of endothelium was assessed qualitatively by the degree of relaxation caused by acetylcholine (10 μM) after precontraction with PE. If there was any degree of relaxation in endothelium-denuded vessels, the rings were eliminated.

Statistical analysis

Data are presented as Means ± SEM. The IC50 values (i.e., the concentration of the agonist or active ingredients (vitexin, procyanidin B_2 and isoquercetine) Non-linear regression analysis was used to determine the concentration–response curves that resulted in a 50% reduction in maximal relaxant responses using GraphPad Prism™ software, version 6.0 (GraphPad Software, USA).

Results

Effect of Vitexin on Isolated Rat's Aorta

1. Effects of L-Name, methylene blue, and Indomethacin on vitexin-induced relaxation in aortic rings

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Cumulative dose-response curves (DRCs) representing the experiments for the effect of vitexin on isolated rat's aortic rings preincubated with L-Name, methylene blue and indomethacin, precontracted with PE are shown in figure (1). L-Name caused a highly significant inhibition in the vitexin induced-vasorelaxation ($P<0.001$) at concentrations ($1*10$-4 to $3*10^{-4}$M), ($P<0.01$) at $3*10^{-5}$ M, and ($P<0.05$) at concentration $2*10^{-5}$ M, with LogIC50 of -4.702 (CI 95% from -5.066 to -4.339). The Emax was greatly reduced to only 4.60% as compared to the control (Emax 58.56%). Similarly, the vasorelaxant effect of vitexin in aorta pretreated with methylene blue has been non-significantly inhibited with LogIC50 of -5.121 (CI 95% from -7.544 to -2.699) with Emax 22.13% whereas in the presence of indomethacin, the Emax was mildly and non-significantly decreased from 58.56% in the control to 49.03% in (table 1).

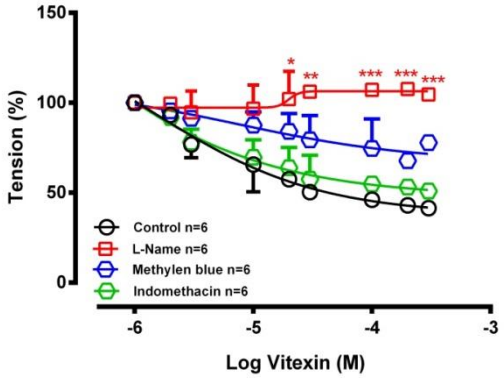

Figure 1: Cumulative dose-response curves for the relaxant effects of vitexin on aorta in absence (control) and those preincubated with L-NAME, methylene blue and indomethacin, and precontracted with PE.

Table 1: The LogIC50, (LogIC50 of 95% CI) and Emax ± SEM induced by vitexin in aorta pretreated with L-NAME, methylene blue and indomethacin.

	Vitexin			
Treatments	**Control**	**L-NAME**	**Methylene Blue**	**Indomethacin**
LogIC50	-5.858	-4.702	-5.121	-6.809
LogIC50 of 95%	-12.47 To 0.7569	-5.066 To -4.339	-7.544 To -2.699	-17.81 To -4.196
***E*max ± SEM%**	58.56 ± 3.29	4.60 ± 0.22	22.13 ± 1.20	49.03 ± 5.45

2. Effect of pre-treatment of aortic rings with K^+ channel blockers on vitexin-induced relaxation

Dose response curves for the relaxant effects of vitexin on aortic rings in absence and presence of different K^+ channel blockers prior to precontraction with PE are shown in figure (2). In the presence of TEA, the DRC of vitexin shifts slightly to the right as compared to the control. The LogIC50 was -5.751 (CI 95% from -7.690 to -3.812). Thus, the Emax was inhibited from 58.56 to 38.73%. In contrast, 4-AP, GLIB and BaCl2 produced a non-significant relaxation with LogIC50 of -6.359 (CI 95% from -9.414 to -3.304), -5.468 (CI 95% from -7.927 to -3.010) and -4.929 (CI 95% from -5.950 to -3.908) and the percentages of relaxation were increased from 58.56% in control to 63.50%, 71.87% and 91.07% respectively. (Table 2).

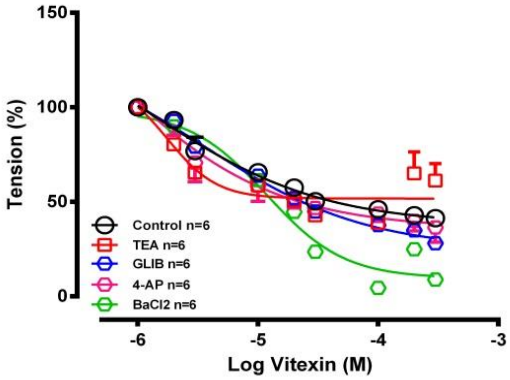

Figure 2: The relaxing effects of vitexin on the aorta in the absence and present of TEA, GLIB, BaCl2, 4-AP, and precontracted with PE are shown as cumulative dose-response curves.

Table 2: The LogIC50, (LogIC50 of 95% CI) and Emax ± SEM induced by vitexin in aorta pretreated with TEA, GLIB, $BaCl_2$ and 4-AP.

	Vitexin				
Treatments	**Control**	**TEA**	**GLIB**	**BaCl2**	**4-AP**
LogIC50	-5.858	-5.751	-5.468	-4.929	-6.359
LogIC50 of 95%	-12.47 To 0.7569	-7.690 To -3.812	-7.927 To -3.010	-5.950 To -3.908	-9.414 To -3.304
***E*max ± SEM%**	58.56 ± 3.29	38.73 ± 0.96	71.87 ± 1.22	91.07 ± 0.51	63.50 ± 1.52

3- Effect of Nifedipine pre-incubation of aortic rings on vitexin-induced relaxation

Common DRCs for control purposes and nifedipine preincubated aortic rings against PE-induced contraction are shown in figure (3). The aortic DRC induced by vitexin was significantly inhibited in the presence of nifedipine at $3*10^{-5}$ to $3*10^{-4}$ M of vitexin. Thus, the Emax was significantly inhibited from 58.56 to only 11.60%. The LogIC50 was -5.628 (CI 95% from -5.809 to -5.447) in comparison to the control (table 3).

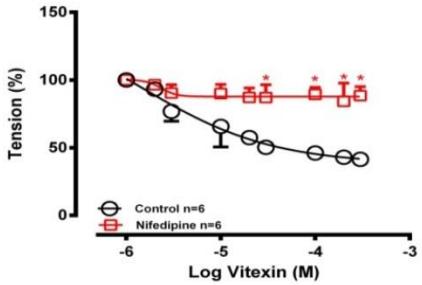

Figure 3: The relaxant effects of vitexin on the aorta in the absence of nifedipine and those preincubated with nifedipine and precontracted with PE are shown as cumulative dose-response curves.

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Table 3: The LogIC50, (LogIC50 of 95% CI) and Emax ± SEM induced by vitexin in aorta pretreated with nifedipine.

	Vitexin	
Treatments	**Control**	**Nifedipine**
LogIC50	-5.858	-5.628
LogIC50 of 95%	-12.47 To 0.7569	-5.809 To -5.447
***E*max ± SEM%**	58.56 ± 3.29	11.60 ± 1.86

4. The role of the endothelium in vitexin-induced aortic relaxation in rats

The DRCs on the relaxation effects of vitexin on PE-precontracted intact and denuded endothelium of aortic rings are shown in figure (4). Denuded endothelium aortic rings exhibited a considerable non-significant inhibition in the dose response curve. In denuded aorta, the LogIC50 was -5.318 (LogIC50 of 95% CI from -8.061 to -2.576), whereas in intact aorta, LogIC50 was -5.858 (LogIC50 of 95% CI from -12.47 to 0.7569). However, the Emax for denuded aorta was reduced from 58.56 ± 3.29% in the control to 25.53% in denuded aorta (table 4).

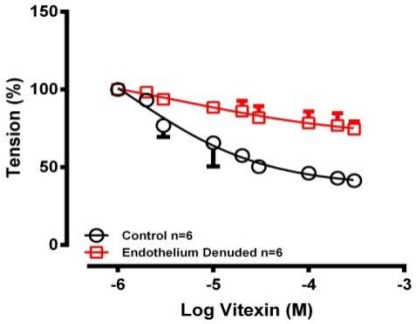

Figure 4: The relaxant effects of vitexin on intact and endothelium-denuded aorta precontracted with PE were studied using cumulative dose-response curves.

Table 4: The LogIC50, (LogIC50 of 95% CI) and Emax ± SEM induced by vitexin in intact and denuded-endothelium aorta.

	Vitexin	
Treatments	**Control**	**Endothelium Denuded**
LogIC50	-5.858	-5.318
LogIC50 of 95%	-12.47 To 0.7569	-8.061 To -2.576
***E*max ± SEM%**	58.56 ± 3.29	25.53 ± 1.36

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Effect of Procyanidin B_2 on Isolated Rat's Aorta

1. **Effect of L-NAME pretreatment on Procyanidin B_2 induced relaxation in aortic rings**

   Figure (5) the DRCs for the effects of L-NAME on Procyanidin B_2 induced relaxation in aortic rings precontracted with PE. Pretreatment with L-NAME considerably enhanced relaxation in precontracted aortic rings with LogIC50 of -5.128 (CI 95% from -7.220 to -3.035) in comparison to the control group. As a result, Emax has risen to 167.25 percent from 137.49 percent (table 5).

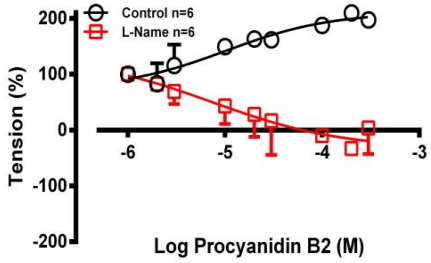

Figure 5: Cumulative dose-response curves for the relaxant effects of Procyanidin B_2 on the aorta in the absence of L-NAME and those precontracted with PE.

Table 5: The LogIC50, (LogIC50 of 95% CI) and Emax±SEM induced by Procyanidin B_2 in aorta pretreated with L-NAME.

	Procyanidin B2	
Treatments	**Control**	**L-NAME**
LogIC50	-4.990	-5.128
LogIC50 of 95%	-10.24 To -0.2636	-7.220 To -3.035
***E*max ± SEM%**	137.49 ± 2.61	167.25 ± 1.04

2- **Effect of Nifedipine pre-incubation on Procyanidin B_2 induced relaxation in aortic rings**

Figure (6) DRCs depict the effect of nifedipine on the procyanidin B_2 induced relaxation of aortic rings precontracted with PE. The DRC of nifedipine revealed the presence of a considerable inhibition in relaxation curve as compared to the control. Thus, the Emax was reduced from 81.44% in the control to 34.80%, with LogIC50 of -3.668 (CI 95% from -9.749 to - 2.413) (table 6).

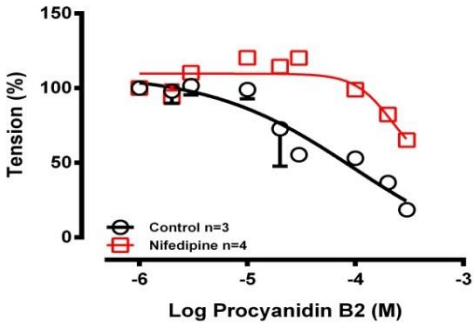

Figure 6: Procyanidin B_2 relaxant effects on the aorta in the absence of nifedipine (control) and those preincubated with nifedipine and precontracted with PE.

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Table 6: The LogIC50, (LogIC50 of 95% CI) and Emax ± SEM induced by Procyanidin B2 in aorta pretreated with nifedipine.

	Procyanidin B2	
Treatments	**Control**	**Nifedipine**
LogIC50	-4.021	-3.668
LogIC50 of 95%	-8.026 To -0.01652	-9.749 To -2.413
***E*max ± SEM%**	81.44 ± 1.94	34.80 ± 3.0

Effect of Isoquercetine on Isolated Rat's Aorta

1. **Effect of L-Name and Indomethacin on Isoquercetine-induced relaxation in the aortic rings**

   The relaxation curves for the control, indomethacin, and L-NAME preincubated aortic rings against PE pre-contraction are shown in figure (7). Pretreatment of the aortic rings with indomethacin showed significant ($P<0.01$) increase in relaxation at doses ($1*10^{-5}$ to $3*10^{-4}$ M) with LogIC50 of -4.348 (CI 95% from -8.119 to -0.5771). with increased Emax to 181.66%. On the other hand, preincubation of the aorta with L-NAME caused a significant ($P<0.05$) inhibition in relaxation at concentrations ($2*10^{-6}$, $3*10^{-6}$, $3*10^{-5}$ and $3*10^{-4}$ M) with LogIC50 of 3.764 (CI 95% from 6.430 to 1.098) with reduction of Emax from 65.39% to 23.51% (table 7).

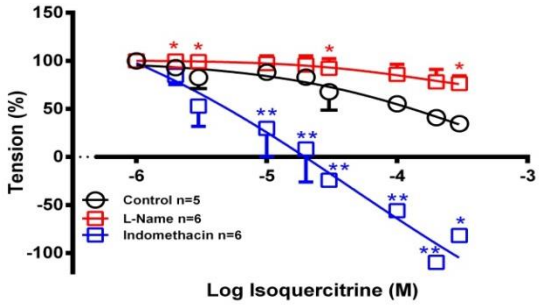

Figure 7: Cumulative dose-response curves for the relaxant effects of isoquercetine on aortic rings in the absence of L-NAME and indomethacin, precontracted with PE, and those preincubated with L-NAME and indomethacin.

Table 7: The LogIC50, (LogIC50 of 95% CI) and Emax ± SEM induced by isoquercetine in aorta pretreated with L-NAME and indomethacin.

	Isoquercetine			
Treatments	**Control**	**L-NAME**		**Indomethacin**
LogIC50	-3.401	3.764		-4.348
LogIC50 of 95%	-15.11 To 8.307	6.430 To 1.098		-8.119 To -0.5771
***E*max ± SEM%**	65.39 ± 5.79	23.51 ± **1.32**		181.66 ± 1.88

2. Effect of a K^+ channel blocker on isoquercetine-induced relaxation in the aortic rings

Figure (8) show DRCs for the effect of **isoquercetine** on PE-precontracted aorta preincubated with GLIB. The DRC of GLIB treated rings showed a non-significant increased relaxation as compared to the control curve. Consequently, the Emax increased from 65.39 to 99.23%, with a LogIC50 of -4.387 (CI 95% from -5.478 to -3.296) compared to that of control (table 8).

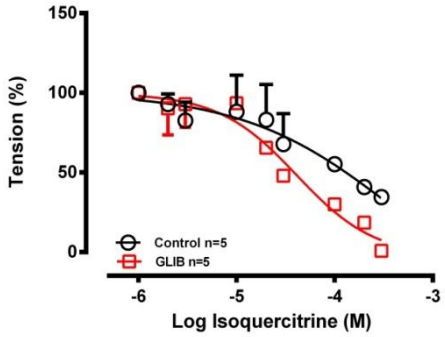

Figure 8: Cumulative dose-response curves for isoquercetine relaxant effects on the aorta in the absence of GLIB (control) and those preincubated with GLIB and precontracted with PE.

Table 8: The LogIC50, (LogIC50 of 95% CI) and Emax ± SEM induced by isoquercetine in aorta pretreated with GLIB.

	Isoquercetine	
Treatments	**Control**	**GLIB**
LogIC50	-3.401	-4.387
LogIC50 of 95%	-15.11 To 8.307	-5.478 To -3.296
***E*max ± SEM%**	65.39 ± 5.79	99.23 ± 0.54

3. Effect of nifedipine pre-incubation of aortic rings on isoquercetine-induced relaxation

Relaxation curves for the control and nifedipine preincubated aortic rings against PE pre-contracted are shown in figure (9). The DRC of aortic rings preincubated with nifedipine revealed the enhancement rather than the inhibition of relaxation as compared with the control, with Emax increased from 65.39 to 87.24%, and IC50 was -4.952 (CI 95% from -8.651 to -1.253) as shown in table (9).

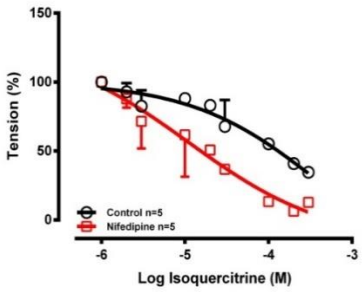

Figure 9. Cumulative dose-response curves of isoquercetine relaxant effects on the aorta in the absence of nifedipine and precontraction with PE in the absence of nifedipine.

Table 9. The LogIC50, (LogIC50 of 95% CI) and Emax ± SEM induced by isoquercetine in aortic rings pretreated with nifedipine.

	Isoquercetine	
Treatments	**Control**	**Nifedipine**
LogIC50	-3.401	-4.952
LogIC50 of 95%	-15.11 To 8.307	-8.651 To -1.253
***E*max ± SEM%**	65.39 ± 5.79	87.24 ± 1.83

Discussion

The current work found that pretreatment of aortic rings with L-NAME, an inhibitor of NO production, dramatically reduced the relaxant effects of vitexin and isoquercetine in PE precontracted aortas, implying that this relaxant action is mediated by endothelial NO release. Similar observation was made by (19) using the same inhibitor. Pretreatment of the aortic rings with L-NAME, on the other hand, did not significantly improve the relaxing effects of procyanidin B2. This contradicted the findings of (20), which showed that procyanidins in *Crataegus* extract caused endothelium-dependent vasorelaxation. Vitexin-induced relaxation in aortic smooth muscle was also aided by endothelial cGMP. Methylene blue (a cGMP inhibitor) was used to confirm this, as it prevented induced relaxation. Similarly, NO relaxes smooth muscle cells in the rat aortic rings by activating guanylate cyclase and producing the second messenger cGMP, which lowers free intracellular Ca^{2+} and its output, resulting in smooth muscle cell relaxation. This was proven by (21) who discovered that cGMP stimulates intracellular effectors such as PKG, causing intracellular Ca^{2+} to decrease and actin and myosin filaments to dissociate, resulting in smooth muscle cell relaxation. Furthermore, the findings of (22) revealed that cGMP plays a significant anti-contraction role in euscaphic acid-induced aortic smooth muscle relaxation (active component of C. aronia).

The relaxing effect of vitexin in conjunction with endothelial cell synthesis of prostacyclin (PGI_2) was confirmed in the aorta by preincubation with indomethacin, a cyclooxygenase inhibitor that decreased relaxation responses. This demonstrates the critical role of PGI_2 in induced relaxation, which is inhibited by the PGI_2 blocker indomethacin. Prostacyclin has a comparable impact on the rat aorta in response to quercetin (19) and euscaphic acid (22). Pretreatment of the aortic rings with indomethacin, on the other hand, greatly increased the relaxing effects generated by isoquercetine. The vasorelaxant effect of denuded endothelial aorta was significantly reduced. This suggested that the relaxant effect of vitexin is mediated by endothelium and endothelium-derived relaxing substances. Similarly, (23) found that the butanol fraction of *C. aronia* generated a significant reduction in endothelium-denuded aortic relaxation. Apigenin's mechanism underlying the relaxation effect of agonist-induced vascular contraction, regardless of endothelial function, was demonstrated using denuded aortic rings (11).

The voltage-gated potassium (Kv) channel had no influence on vitexin-induced relaxation since it increased maximal relaxation in the aortic ring that had been pre-incubated with 4-AP (Kv channel blocker). The temporary rise in cytosolic Ca2+, on the other hand, activates calcium channels in the plasma membrane, allowing extracellular calcium to influx and depolarize the membrane, allowing the Kv channel to hyperpolarize the membrane and enable smooth muscle relaxation (24). Calcium activated potassium (Kca) channel plays an important role in the relaxation mediated by vitexin, since TEA (Kca channel blocker) inhibited the induced relaxation, which reflects that the Kca channels playing an important

role in the relaxation induced by vitexin. A similar trend of response was reported for quercetin induced relaxation of coronary arteries (25) and euscaphic acid induced relaxation (19) since both studies indicated the important role of Kca channel in induced relaxation.

The results of the current study indicated that vitexin and isoquercetine-mediated relaxations are not dependent on the activation of K_{ATP} sensitive channels, since the presence of GLIB (K_{ATP} channel blocker) both vitexin and isoquercetine induced relaxant responses in aortic rings were enhanced rather than inhibited. Usually, K_{ATP} channels are stimulated by its openers, such as pinacidil and cromakalim, which causing K^+ influx, hyperpolarization in the smooth muscle cell membrane, and subsequent vasodilation and drop in blood pressure (26). Since euscaphic acid did not activate K_{ATP} channels in rat aortic smooth muscle cells, our findings are consistent with those reported by (19). Similarly, (27) discovered that adding GLIB to *Crataegus* extract did not modify its relaxing activity in the smooth muscle cells of the rat's arteries. The inward-rectifier potassium (Kir) channel was also not involved in vitexin-induced relaxation, as evidenced by pre-incubation of aortic rings with $BaCl_2$ (a Kir channel blocker), which promoted rather than inhibited vitexin-induced relaxation. This is consistent with the findings of (28) who discovered that the Kir channel plays no function in the organization of smooth muscle resting membrane potential and resting tone. Kir channels, on the other hand, are thought to play a key part in determining the cell's resting potential, according to (29) Kir channels are also thought to play a role in the relaxing of the rat's ileum (30).

The relaxant effect of vitexin was significantly reduced when the aorta was pre-incubated with nifedipine (an L-type Ca2+ channel blocker). This clearly indicates that the L-type Ca^{2+} channel plays a critical role in the relaxation induced by vitexin, and that the induced relaxation is dependent on the activation of L-type Ca^{2+} channels, which leads to the activation of K^+ channels, and causes smooth muscle relaxation. This explains why the aorta's contraction is not solely dependent on external Ca^{2+} intake, but also on Ca^{2+} release from intracellular storage. This theory is supported by (31,32), who found that increased cytosolic free Ca^{2+} induced by its release from the SR via ryanodine or IP3 receptors activated K^+ channels, resulting in smooth muscle relaxation. While the L-type Ca2+ channel plays a minor role in procyanidin B_2-mediated relaxation, it was not significantly blocked. Pretreatment of the aortic rings with nifedipine, on the other hand, had no influence on the relaxing effects induced by isoquercetine.

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