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Garcinia cowa Leaf Ethanolic Extract Induces Vasorelaxation Through eNOS/NO/sGC Pathway, Potassium, and Calcium Channels in Isolated Rat Thoracic Aorta

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ABSTRACT

Background: *Garcinia cowa* Roxb. ex Choisy (*G. cowa*) is used in traditional medicine, both for improvement of blood circulation and indigestion, also as an antipyretic and expectorant. **Objective:** This study investigated the vasorelaxant effects and possible mechanisms of action of *G. cowa* leaf ethanolic extract (GCE) on the rat isolated thoracic aorta. **Methods:** The study examined the effects of GCE on isolated rat thoracic aorta, including both endothelium-intact and endothelium-denuded aortic rings, using an organ bath system. Specific inhibitors were used to evaluate the mechanism involved in GCE-induced vasorelaxation. **Results:** GCE (0.01–10 mg/mL) relaxed endothelium-intact aortic rings, that had been pre-contracted with phenylephrine. Removal of the endothelium or pretreatment of endothelium-intact aortic rings with N^{ω}-nitro-L-arginine methyl ester (L-NAME), or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), significantly decreased vasorelaxation induced by the GCE. Indomethacin or propranolol had no effect on the GCE-induced relaxation of the endothelium-intact aortic rings. In endothelium-denuded aortic rings, the relaxation effect of GCE was significantly blocked by 4-aminopyridine (4-AP) and tetraethylammonium (TEA) at the maximum dose of GCE, but not by glibenclamide. In Ca^{2+}-free Krebs solution, GCE (5 and 10 mg/mL) significantly inhibited extracellular Ca^{2+} induced contraction in pre-contracted rings with high KCl levels. **Conclusions:** These findings suggest that GCE exhibits both an endothelium-dependent, which is mediated by an eNOS/NO/sGC pathway, and an endothelium-independent pathway, which involves K_{Ca} and K_V channels opening and extracellular Ca^{2+} influx inhibition. Kaempferol, isovitexin, quercetin, apigenin, luteolin, and amentoflavone might play a role in inducing the vasorelaxant effect of GCE.

Keywords: *Garcinia cowa*, Vasorelaxant effect, Endothelium, K^+ channel, Ca^{2+} channel.

INTRODUCTION

Garcinia cowa Roxb. ex Choisy (*G. cowa*), Thai name Cha muang, is a member of the Clusiaceae family. It is found in the tropical and subtropical countries of West and East Africa, and Southeast Asia.[1] Young leaves of *G. cowa* are consumed as vegetables. In traditional medicine, most parts of this plant have been used for varied purposes. The bark, latex and root are used as an antipyretic agent for the treatment of fever.[2-3] The fruits and leaves are used for improvement of blood circulation, indigestion, and as an expectorant.[3] Some pharmacological properties of the crude extracts have been reported to have activity, such as antitumor-promotion,[4] anti-platelet,[5] antioxidant, antimutagenic,[6] and antibacterial[7] activities. The major compounds found in various extracts of *G. cowa* are xanthones and phloroglucinols.[8] These compounds possess anticancer,[9-11] antibacterial,[12-13] anti-inflammatory, antioxidant,[3] antimalarial,[14] and α-glucosidase inhibitory[1,15] effects, and they exhibit nitric oxide (NO) production.[15] Other compounds also observed in *G. cowa* included depsidones, terpenoids, steroids, and flavonoids.[8]

Although the fruits and leaves of *G. cowa* have been traditionally used to enhance blood circulation, there are insufficient scientific reports to test these therapeutic claims. Previous studies demonstrated that the aqueous extract from the leaves of *G. cowa* induced vasorelaxation through an endothelium-dependent pathway, involved in the production of NO and prostanoids, and an endothelium-independent pathway, by the opening of ATP-sensitive K^+ channels (K_{ATP}). Flavonoids most likely played a role in the vasorelaxant effect of *G. cowa* leaf extract.[16] However, the solvents used in the extraction process are noted to affect both the composition and quantity of secondary metabolites obtained from medicinal plants.[17] Additionally, differences in plant varieties and collection locations may result in distinct compositions of active compounds, with both genetic and environmental factors critically influencing the biosynthesis and accumulation of secondary metabolites.[18] The diversity of active compounds in the extract can result in different mechanisms of action. Furthermore, other mechanisms of action that may be involved in the vasorelaxant effect of *G. cowa* require further study. Therefore, in the present study, we aimed to investigate the vasorelaxant effects and possible mechanisms of action of *G. cowa* ethanolic leaf extract.

MATERIALS AND METHODS

Chemicals and drugs

1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 4-aminopyridine (4-AP), acetylcholine, glibenclamide, indomethacin, nifedipine, N -nitro-L-arginine methyl ester (L-NAME), phenylephrine hydrochloride, propranolol hydrochloride and tetraethylammonium (TEA), were purchased from Sigma (St. Louis, MO, USA). Dimethyl

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sulfoxide (DMSO) was of analytical grade and *purchased from RCI Labscan* (Thailand). Indomethacin, ODQ, nifedipine, glibenclamide and *G. cowa* extract were dissolved in DMSO, and the remainder were dissolved in distilled water.

Plant materials and preparation of the extract

Leaves of *G. cowa* were collected from Chiang Rai Province, Thailand. Botanical identification of the plant was carried out by Assoc. Prof. Rawiwan Charoensup, School of Integrative Medicine, Mae Fah Luang University, Chiang Rai, Thailand. A voucher specimen (MFU-NPR0190) was deposited at the Natural Products Research Laboratory, School of Science, Mae Fah Luang University, Thailand.

Air-dried leaves of *G. cowa* (1.70 kg) were ground into a fine powder. The powder was treated with 95% ethanol three times. The solution was collected, filtered then evaporated using a rotary evaporator at 60 ºC to give 211.29 g (12.42% yield) of *G. cowa* extract (GCE). The extract was stored at -20 ºC until use.

LC-QTOF-MS analysis

The chemical constituent profiles of GCE were characterized by LC-QTOF-MS using an Agilent 1290 Infinity II UHPLC System equipped with an Agilent G6545B QTOF/MS system (Agilent Technologies, Santa Clara, CA, USA). Separation was achieved using an Agilent Poroshell EC-C18 column (150 mm × 2.1 mm, 2.7 µm). The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). The flow rate was set at 0.2 mL/min, and the injection volume was 1 µL. Peak identification was performed in negative ionization modes. Mass spectra ranging from 50 to 1100 m/z were obtained. Fixed collision energies (10, 20, and 40 eV) were used. The gas temperature was set at 300 °C with a flow rate of 10 L/min. The sheath gas temperature, on the other hand, was set at 350 °C with a flow rate of 11 L/min. The nebulizer pressure was set at 35 psi. The voltages of the capillary and nozzle were 3.5 and 1 kV, respectively. Agilent LC-MS-QTOF Mass-Hunter data acquisition software was utilized for data acquisition and analysis.

Animals

Male Wistar rats, weighing 250–300 g, were obtained from the Nomura Siam International Co, Ltd, Thailand. All animals were maintained in a controlled temperature (23–25 °C), with a 12 h light/dark cycle and allowed free access to standard food and water. All animal experimental protocols were approved by the Animal Ethics Committee of Mae Fah Luang University, Thailand, with approval number AR01/61.

Preparation of isolated aortic rings

Rats were anesthetized with thiopental sodium (40 mg/kg, i.p.). The thoracic aorta was carefully removed and placed in a petri dish filled with Krebs Henseleit solution, (118.3 mM NaCl, 25.0 mM $NaHCO_3$, 11.66 mM glucose, 4.7 mM KCl, 1.9 mM $CaCl_2$, 1.18 mM KH_2PO_4, 0.45 mM $MgSO_4 \cdot 7H_2O$, 0.09 mM ascorbic acid and 0.024 mM Na_2EDTA), at 37 °C and aerated with carbogen (95% O_2 and 5% CO_2). The aorta was then cleaned to remove adhering fat and connective tissue before being cut into rings of 4–5 mm in length. The aortic rings were then mounted horizontally between two stainless steel hooks in an organ bath that contains 20 mL of Krebs Henseleit solution. The organ bath chambers were maintained at 37 °C, and continuously supplied with carbogen. Vascular tone was recorded using iWorx FT-104 force transducer (iWorx Systems Inc., Dover, NH, USA), connected to a iWorx IX-214 data recorder (iWorx Systems Inc., Dover, NH, USA). The data were recorded using Labscribe2 software (iWorx Systems Inc., Dover, NH, USA). The aortic rings were equilibrated at a resting tension of 1 g for at least 60 min with a change of the Krebs Henseleit solution every 15 min. In endothelium-denuded rings, the endothelium was mechanically removed by gently rubbing the inner surface of the aorta with a stainless-steel rod. After the equilibration period, all preparations were tested for the presence of a functional endothelium by pre-contracting the vessels with phenylephrine (3 M) and recording the relaxant response to acetylcholine (30 M). Endothelium was considered intact when acetylcholine caused a relaxation response of up to 80% in preparations. When the acetylcholine-induced relaxation was less than 10%, the aortic rings were considered to be denuded functional endothelium. The aortic rings were then allowed to equilibrate for 45 min with a change of the Krebs Henseleit solution every 15 min before commencing the experimental protocols.

Experimental protocols

To study the vasorelaxant effect of GCE, aortic rings with and without endothelium were pre-contracted with phenylephrine (3 µM). Once the contraction stabilized, a concentration-response curve was created by the cumulative addition of the GCE (0.01–10 mg/mL).

To evaluate the mechanism involved in GCE-induced vasorelaxation, various inhibitors were used. The aortic rings were pre-incubated for 30 min with endothelial nitric oxide synthase (eNOS) inhibitor, L-NAME (100 µM); cyclooxygenase (COX) inhibitor, indomethacin (10 µM); soluble guanylyl cyclase (sGC) inhibitor, ODQ (1 µM); nonselective β-adrenergic receptor blocker, propranolol (1 µM); nonselective calcium-activated K^+ channel (K_{Ca}) blocker, TEA (5 mM); voltage-dependent K^+ channel (K_V) blocker, 4-AP (1 mM); or nonspecific K_{ATP} blocker, glibenclamide (10 µM). After incubation, the aortic rings were pre-contracted using phenylephrine (3 µM). Once the contraction reached a plateau, cumulative concentrations of the GCE (0.01–10 mg/mL) were added to the chamber.

To examine whether the relaxation effects of GCE were the result of GCE inhibition of extracellular Ca^{2+} influx into vascular smooth muscle cells (VSMCs), the endothelium-denuded aortic rings were incubated in Ca^{2+}-free Krebs solution for 40 min. The rings were then replaced in Ca^{2+} free and high-KCl (80 mM) Krebs solution for 10 min to open the voltage-operated Ca^{2+} channels (VOCC). A concentration-contraction response was obtained by the cumulative addition of the $CaCl_2$ (0.01–10 mM). The aortic rings were then washed with Ca^{2+}-free Krebs solution and were again depolarized with the Ca^{2+}-free and high-KCl (80 mM) Krebs solution. The rings were then incubated with three concentrations of GCE, (2.5, 5 or 10 mg/mL), the vehicle, or nifedipine (L-type VOCC blocker, 1 µM) for 30 min before $CaCl_2$ (0.01–10 mM) was cumulatively added.

Data and statistical analysis

The data are expressed as mean ± SEM of 6 animals (n = 6). Vasodilator responses of the thoracic aortic rings are expressed as percentage relaxation of vessels from maximal contraction of phenylephrine (3 µM) pre-contracted levels. GraphPad Prism software (version 6.0) was used to fit the original concentration-response curve, allowing for the analysis of the concentration of GCE that caused 50% of the maximal relaxation (EC_{50}) and the maximal relaxation (E_{max}) values. All statistical analyses were performed by using SPSS software (version 26.0). Differences in means between the two groups were assessed by paired or unpaired Student's *t*-test. Multiple comparisons between experimental groups were analyzed using one-way ANOVA followed by Tukey test. A $p < 0.05$ was considered statistically significant.

RESULTS

Phytochemical constituents of GCE

The chemical constituent profile of GCE was carried out by LC-QTOF-MS analysis, with the identified compounds presented in Table 1. LC-QTOF-MS analysis demonstrated that GCE contains

Table 1: Compounds identified in GCE by LC-QTOF-MS using negative ionization mode.

No.	Proposed Compounds	Molecular Formula	RT (min)	Molecular Weight	Diff (DB, ppm)
1	Hydroxycitric acid	$C_6H_8O_8$	2.301	208.0216	1.63
2	Citric acid	$C_6H_8O_7$	2.393	192.0265	0.53
3	Cyanidin 3-(2"-glucuronosylglucoside)	$C_{27}H_{28}O_{17}$	10.965	624.1337	-1.61
4	Formononetin 7-(2-p-hydroxybenzoylglucoside)	$C_{29}H_{26}O_{11}$	11.969	550.1467	1.45
5	Isomangiferin	$C_{19}H_{18}O_{11}$	12.826	422.0838	2.52
6	Kaempferol 3-alpha-D-galactoside	$C_{21}H_{20}O_{11}$	13.428	448.0997	1.95
7	Isovitexin 4'-O-rhamnopyranoside	$C_{27}H_{30}O_{14}$	14.858	578.1629	0.70
8	Apigenin 7-neohesperidoside	$C_{27}H_{30}O_{14}$	15.127	578.1625	1.06
9	Emodin 8-glucoside	$C_{21}H_{20}O_{10}$	15.613	432.1048	2.01
10	Scutellarein 7-glucuronide	$C_{21}H_{18}O_{12}$	16.228	462.0787	2.52
11	Myricetin 3-(3'''-6'''-diacetylglucosyl)-(1->4)-(2",3"-diacetylrhamnoside)	$C_{35}H_{38}O_{21}$	16.936	794.1887	2.31
12	Eriodictyol 7-(6-galloylglucoside)	$C_{28}H_{26}O_{15}$	17.172	602.1260	1.86
13	Luteolin 3'-methyl ether 7-glucuronide-4'-rhamnoside	$C_{28}H_{30}O_{16}$	17.222	622.1522	1.95
14	Kaempferol 3-(2"-pcoumarylglucoside)	$C_{30}H_{26}O_{13}$	17.247	594.1365	0.80
15	Quercetin 3-(3",4"-diacetylrhamnosyl)-(1->6)-glucoside	$C_{31}H_{34}O_{18}$	17.319	694.1726	2.74
16	Luteolin 7-(6"-acetylglucoside)	$C_{23}H_{22}O_{12}$	17.415	490.1106	1.15
17	Casticin 3'-glucoside	$C_{25}H_{28}O_{13}$	17.448	536.1548	-3.41
18	Apigenin 7-(4"-E-p-coumarylglucoside)	$C_{30}H_{26}O_{12}$	17.510	578.1414	1.81
19	Cyanidin 3-(6"-acetylglucoside)	$C_{23}H_{22}O_{12}$	18.427	490.1122	-2.10
20	Amentoflavone	$C_{30}H_{18}O_{10}$	18.576	538.0879	3.98
21	Garcimangosone C	$C_{23}H_{24}O_7$	21.336	412.1518	0.90

Table 2: EC_{50} and E_{max} of GCE-induced relaxation in endothelium-intact (E+) and endothelium-denuded (E-) aortic rings in the absence (control) or presence of various inhibitors.

	EC_{50} (mg/mL)	E_{max} (%)
GCE (E+)	2.10 ± 0.27	102.07 ± 1.59
GCE (E-)	5.50 ± 0.60[a]	93.35 ± 3.85
Control	2.19 ± 0.45	105.09 ± 3.16
+ L-NAME	3.76 ± 0.15[c]	104.71 ± 3.54
Control	2.21 ± 0.65	104.85 ± 7.61
+ Indomethacin	1.43 ± 0.32	110.07 ± 4.85
Control	1.65 ± 0.23	108.43 ± 6.40
+ ODQ	3.86 ± 0.26[c]	98.15 ± 3.89
Control	2.16 ± 0.57	102.10 ± 2.20
+ Propranolol	2.68 ± 0.67	107.30 ± 5.99
Control	5.41 ± 0.34	87.58 ± 2.81
+ TEA	7.65 ± 0.89[b]	68.20 ± 3.24[c]
Control	4.73 ± 0.23	95.54 ± 1.74
+ 4-AP	5.05 ± 0.22[b]	85.90 ± 1.78[c]
Control	5.01 ± 0.29	95.09 ± 1.66
+ Glibenclamide	4.36 ± 0.31	97.35 ± 2.50

Data are expressed as mean ± SEM (n = 6). EC_{50} is the concentration of GCE that induced 50% maximal relaxation. E_{max} is the maximal relaxation of aortic rings expressed as percentage relaxation of vessels from maximal contraction of phenylephrine (3 μM) pre-contracted levels.
[a] $p < 0.001$, compared to the endothelium-intact rings.
[b] $p < 0.05$, compared to the control.
[c] $p < 0.01$, compared to the control.

flavonoids and xanthones, including kaempferol, isovitexin, apigenin, scutellarein, myricetin, eriodictyol, luteolin, quercetin, amentoflavone, isomangiferin, and garcimangosone C.

Vasorelaxant effect of GCE

GCE (0.01–10 mg/mL) produced relaxation in aortic rings with or without endothelium, pre-constricted with phenylephrine in a concentration-dependent manner. GCE induced relaxation in endothelium-intact aortic rings with an EC_{50} of 2.10 ± 0.27 mg/mL and an E_{max} of 102.07 ± 1.59%. While in endothelium-denuded rings, GCE induced relaxation less than endothelium-intact rings, with an EC_{50} value of 5.50 ± 0.60 mg/mL and an E_{max} of 93.35 ± 3.85% (Figure 1 and Table 2).

Effects of various inhibitors on GCE-induced vasorelaxation

As illustrated in Figure 2, pretreatment with L-NAME significantly reduced the vasorelaxant effect of GCE with an EC_{50} value of 3.76 ± 0.15 mg/mL and an E_{max} value of 104.71 ± 3.54%, compared to the control group (EC_{50} = 2.19 ± 0.45 mg/mL and E_{max} = 105.09 ± 3.16%). However, pre-incubation with indomethacin did not inhibit GCE-induced endothelium-dependent relaxation, with an EC_{50} of 1.43 ± 0.32 mg/mL and an E_{max} of 110.07 ± 4.85% compared to the control group (EC_{50} = 2.21 ± 0.65 mg/mL and E_{max} = 104.85 ± 7.61%) (Table 2).

In order to investigate whether GCE directly induced vasorelaxation via the activation of sGC, the endothelium-intact aortic rings were incubated with sGC inhibitor, ODQ (1 μM) before pre-contracting with phenylephrine (3 μM). Pre-incubation with ODQ significantly reduced the relaxation effect induced by GCE in endothelium-intact aortic rings (EC_{50} = 3.86 ± 0.26 mg/mL and an E_{max} = 98.15 ± 3.89%) compared to the control group (EC_{50} = 1.65 ± 0.23 mg/mL and an E_{max} = 108.43 ± 6.40%) (Figure 3 and Table 2).

To determine whether the vasorelaxation effects of GCE were mediated by β-adrenergic receptors, the endothelium-intact aortic rings were pre-incubated with nonselective β-adrenergic receptor blocker, propranolol. Pre-incubation with propranolol did not alter the GCE-induced relaxation (Figure 4 and Table 2).

To test involvement of K^+ channel in GCE-induced relaxation, various K^+ channel blockers were used. As shown in Figure 5, pre-incubation with the K_{ATP} blocker glibenclamide had no effect on the GCE-induced relaxation. However, pretreatment with TEA, K_{Ca} blocker or 4-AP, K_V blocker significant inhibited the GCE-induced relaxation at the maximum dose of GCE with an E_{max} value of 68.20 ± 3.24% (EC_{50} = 7.65 ± 0.89 mg/mL) and an E_{max} value of 85.90 ± 1.78% (EC_{50} = 5.05 ± 0.22 mg/mL), respectively (Table 2).

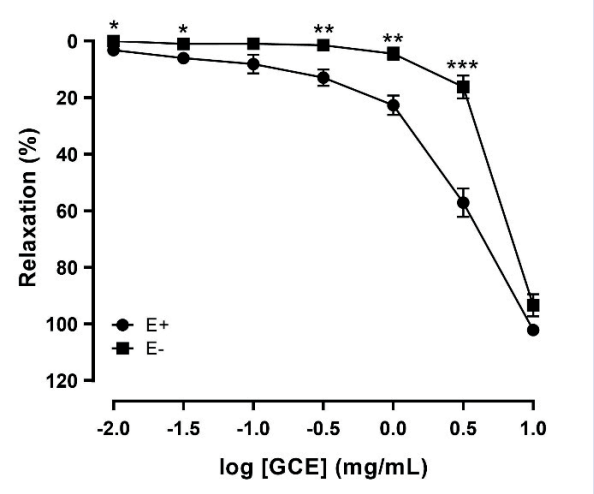

Figure 1: The effect of GCE on phenylephrine (3 μM) pre-contracted endothelium-intact (E+) or endothelium-denuded (E-) aortic rings. Data are expressed as mean ± SEM (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the endothelium-intact aortic rings.

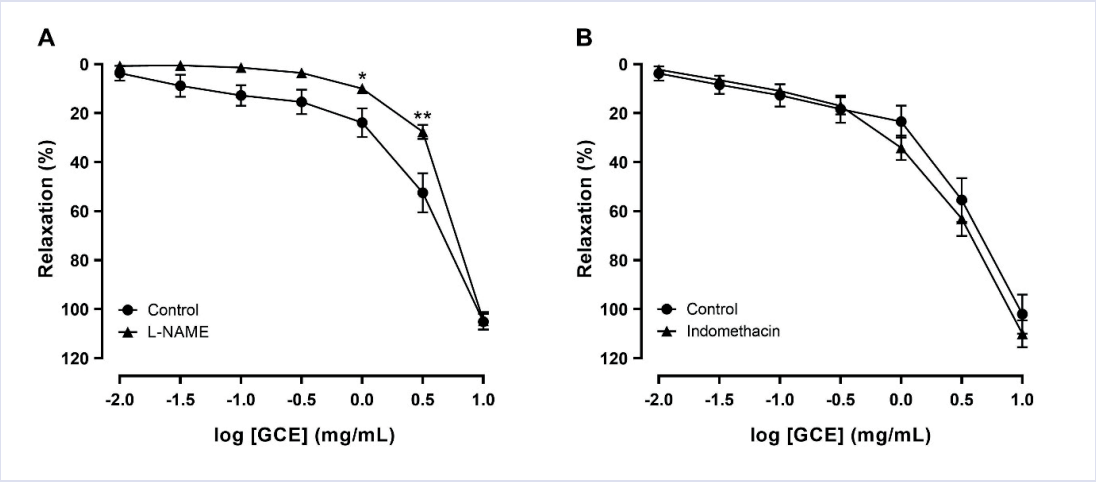

Figure 2: The vasorelaxant response induced by GCE (0.01–10 mg/mL) in endothelium-intact aortic rings pre-contracted with phenylephrine (3 μM) in the absence (control) or presence of 100 μM L-NAME (A) and 10 μM indomethacin (B). Data are expressed as mean ± SEM (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the control.

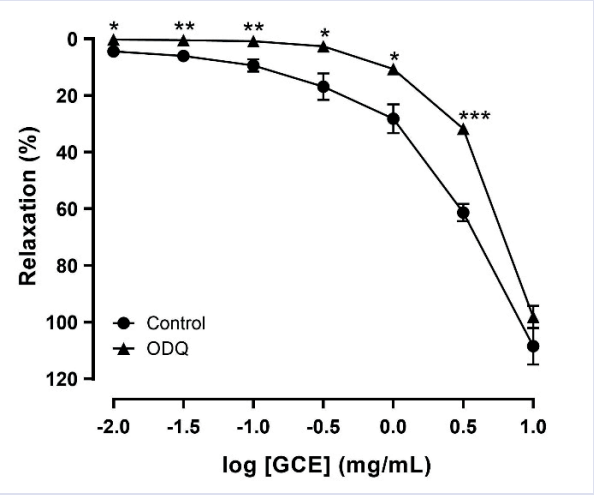

Figure 3: The vasorelaxant response induced by GCE (0.01–10 mg/mL) in endothelium-intact aortic rings pre-contracted with phenylephrine (3 μM) in the absence (control) or presence of ODQ (1 μM). Data are expressed as mean ± SEM (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the control.

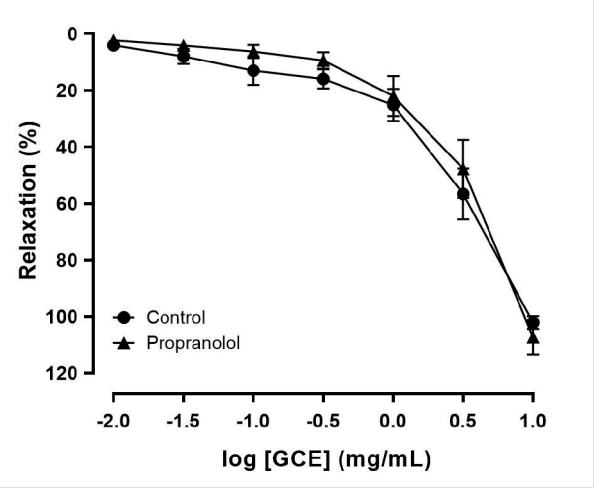

Figure 4: The vasorelaxant response induced by GCE (0.01–10 mg/mL) in endothelium-intact aortic rings pre-contracted with phenylephrine (3 μM) in the absence (control) or presence of propranolol (1 μM). Data are expressed as mean ± SEM (n = 6).

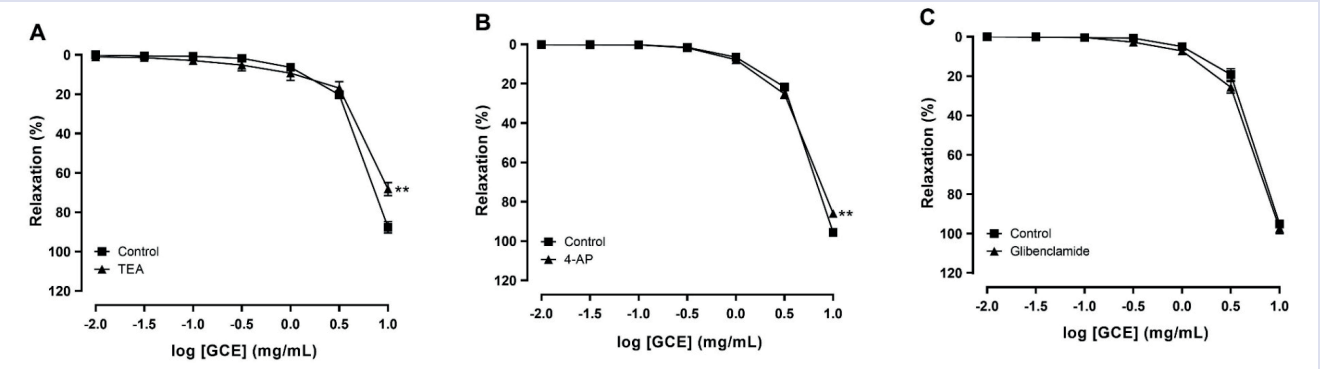

Figure 5: The vasorelaxant response induced by GCE (0.01–10 mg/mL) in endothelium-intact aortic rings pre-contracted with phenylephrine (3 μM) in the absence (control) or presence of 5 mM TEA (A), 1 mM 4-AP (B), or 10 μM glibenclamide (C). Data are expressed as mean ± SEM (n = 6). $^{**}p < 0.01$ compared to the control.

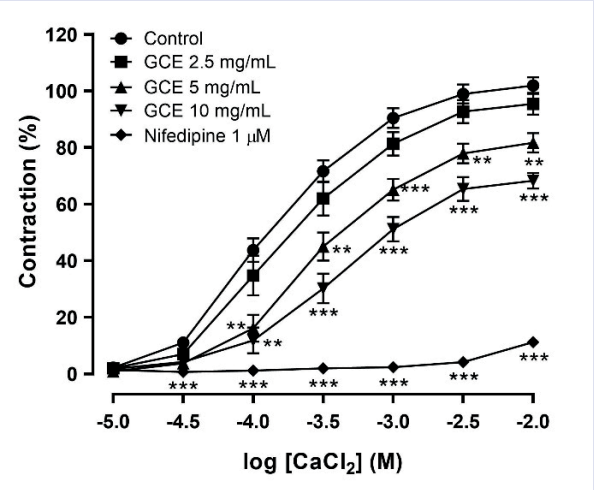

Figure 6: The effect of GCE (2.5, 5 or 10 mg/mL), or nifedipine (1 μM) on $CaCl_2$-induced contraction in endothelium-denuded aortic rings pretreated with 80 mM KCl. Data are expressed as mean ± SEM (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the control.

Effects of GCE on extracellular Ca^{2+}-induced vasoconstriction

In order to determine whether the GCE-induced relaxation is mediated by inhibition of extracellular Ca^{2+} influx, the tissues were depolarized, using a high concentration of KCl (80 mM). GCE (5 and 10 mg/mL) and nifedipine produced a rightward shift of $CaCl_2$ curves and decreased the maximum contraction induced by 10 mM $CaCl_2$ to 81.66 ± 3.40%, 68.28 ± 2.78% and 11.21 ± 1.70%, respectively (Figure 6).

DISCUSSION

In the present study, GCE induced relaxation in rat thoracic aortic rings, via both an endothelium-dependent and an endothelium-independent pathway. The mechanisms responsible for the vasorelaxant effects involve the mediation of an eNOS/NO/sGC pathway, the opening of K^+ channels, and the inhibition of extracellular Ca^{2+} influx.

The vascular endothelium is crucial in regulating the cardiovascular system, particularly in controlling vasomotor tone. The vascular endothelium regulates vasomotor tone through the synthesis or release of vasodilator substances like nitric oxide (NO) and prostacyclin (PGI_2).[19] In endothelial cells, NO is produced from L-arginine through the catalytic action of eNOS. NO then diffuses into vascular smooth muscle cells and simulates sGC, producing the cyclic guanosine phosphate (cGMP)-mediated vasorelaxation.[20] The rise in intracellular cGMP activated cGMP-dependent protein kinases leads to relaxation of vascular smooth muscle cells, by a decrease in intracellular Ca^{2+} concentration.[21] PGI_2 is mainly synthesized from arachidonic acid through the catalytic action of the enzyme COX.[20] Binding of PGI_2 to its receptors, which are located on the membrane of VSMCs, stimulates adenylate cyclase. Adenylate cyclase induces conversion of the adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP), which will then activate the protein kinase A, this causing its vasorelaxant effects.[22] The current investigation revealed that GCE induced vasorelaxation in endothelium-intact aortic rings in a concentration-dependent manner. However, this vasorelaxant effect significantly diminished in endothelium-denuded aortic rings, indicating the involvement of endothelium-derived relaxing factors, such as NO and PGI_2. The endothelium-intact aortic rings were pre-incubated with the eNOS inhibitor, L-NAME, or with COX inhibitor, indomethacin, before being pre-contracted with phenylephrine to investigate the vasorelaxant effect of GCE on the eNOS/NO or COX pathways. L-NAME, an inhibitor of eNOS, significantly reduced the vasorelaxant effect of GCE, which indicates that NO might contribute to the relaxation induced by GCE. To further investigate the involvement of NO, ODQ, an sGC inhibitor, was used. The result revealed that ODQ significantly reduced the GCE-induced vasorelaxation. This suggests that GCE induced relaxation, mediated through the endothelium-dependent eNOS/NO/sGC signaling pathway. On the other hand, the presence of a nonselective COX inhibitor, indomethacin, did not alter the vasorelaxant activity of GCE. These findings indicated that the vasorelaxation induced by GCE was not related to PGI_2 production. However, previous research has proposed the involvement of endothelial synthesis of prostanoids as part of the vasorelaxant effect of *G. cowa* aqueous leaf extract.[16] Variations in activity could be attributed to the existence of different bioactive compounds in *G. cowa* extract.

The results of the current study revealed that removal of endothelium did not completely abolish GCE-induced vasorelaxation, suggesting that GCE could act directly at the VSMCs such as β_2-adrenergic receptor to produce vasodilatory activity. The β_2-adrenergic receptor, a $G\alpha_s$-protein-coupled receptor (GPCR), is only located on the membrane of VSMCs.[23] Activation of the β-adrenergic receptor stimulates adenylyl cyclase activity, which catalyzes the conversion of ATP to cAMP. This subsequently activates protein kinase A and leads to vasodilation.[24] We used propranolol, a nonselective β-adrenergic receptor blocker, to examine the role of the β-adrenergic receptor on the vasorelaxant effects of the GCE. The result showed that propranolol did not modify GCE-induced vasorelaxation, indicating that GCE did not act via the β-adrenergic receptor on VSMCs.

Ion channels such as K^+ channels and Ca^{2+} channels in the plasma membrane of VSMCs are crucial for regulating vascular tone.[25] The opening of K^+ channels in VSMCs causes membrane hyperpolarization, resulting in arterial dilation.[26] Four distinct types of K^+ channels in arterial smooth muscle have been identified, namely K_{Ca}, K_V, K_{ATP} and K_{ir}.[27] In order to investigate the involvement of K^+ channels in the GCE-induced vasorelaxation, various types of K^+ channel blocker, TEA (5 mM), 4-AP (1 mM) and glibenclamide (1 μM) were used. This study demonstrated that the relaxation of endothelium-denuded aortic rings induced by the maximum dose of GCE was significantly attenuated by the K_{Ca} channel blocker, TEA and the K_V channel blocker, 4-AP. However, the K_{ATP} channel blocker, glibenclamide, had no effect on the GCE-induced relaxation. Therefore, the activation of K_{Ca} and K_V channels is partially involved in the vasorelaxation induced by GCE. The results of this study differ from previous findings. Recent research revealed that the leaves of *G. cowa* extract induced vasorelaxation by activating K_{ATP}.[16] The differences in channel activation and subsequent vasorelaxation could be explained by the presence of various bioactive compounds in the *G. cowa* extract.

The trigger of vascular smooth muscle contraction is due to the influx of Ca^{2+} through Ca^{2+} channels in the plasma membrane and its release from intracellular stores.[28] The increased extracellular K^+ conductance and the depolarization of the VSMCs membrane activates the VOCC and causes the extracellular Ca^{2+} to influx into the cytosol.[23] Opening of the K^+ channels in VSMCs results in hyperpolarization. Hyperpolarization closes the VOCC and inhibits extracellular Ca^{2+} influx, leading to vasorelaxation.[28] In order to investigate the involvement of GCE on the VOCC, the aortic rings were incubated with GCE in a Ca^{2+}-free Krebs solution and $CaCl_2$ was added to the organ bath chamber in a cumulative concentration. Results showed that the addition of $CaCl_2$ caused a concentration-dependent vasoconstriction response. Nifedipine, an L-type voltage-gated calcium channel blocker, almost completely abolished the $CaCl_2$-induced contraction. The $CaCl_2$-induced contraction was also significantly inhibited by GCE, indicating that GCE can inhibit extracellular Ca^{2+} influx via VOCC and cause vasorelaxation.

In the present study, our results indicate that flavonoids and xanthones, including kaempferol, isovitexin, apigenin, scutellarein, myricetin, eriodictyol, luteolin, quercetin, amentoflavone, isomangiferin, and garcimangosone C were detected in GCE by LC-QTOF-MS analysis. It is probable that flavonoids identified in the extract responsible for vasorelaxation. Several evidences indicating that flavonoids exhibit vasorelaxant properties.[29-30] In previous studies, flavonoids, kaempferol, vitexin, and isovitexin have been identified as key compounds responsible for the vasorelaxation effect of *G. cowa* aqueous leaf extract.[16] However, our current study has yielded a different outcome. The results of the present study revealed the presence of kaempferol and isovitexin, but we did not detect vitexin in GCE. The vasorelaxant action of GCE may be attributed to kaempferol and isovitexin, which is consistent with previous research.[16] Moreover, we identified additional active substances that may also contribute to the vasorelaxation effect of GCE. The LC-QTOF-MS analysis of GCE detected other flavonoids such as quercetin, apigenin, luteolin, and amentoflavone, and these compounds may play a role in inducing vasorelaxation.

Several studies have investigated the vasorelaxant effects of quercetin, revealing multiple mechanisms pathways. It promotes vasorelaxation via NO- and prostaglandin-dependent pathways,[31] increases NO production, which in turn elevates cGMP levels,[32] and

inhibits Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels while increasing voltage-gated K^+ channel activity in smooth muscle cells.[33] Additionally, apigenin has been reported to exhibit vasorelaxation activity that involves both endothelium-dependent and endothelium-independent mechanisms. The reported mechanisms underlying the vasorelaxant activity of apigenin include the production of NO and cGMP, inhibition of extracellular Ca^{2+} influx,[34] activation of K^+ channels,[29,34] and stimulation of transient receptor potential vanilloid 4 cation channels.[35] There is evidence that luteolin and amentoflavone have vasorelaxation effects. Luteolin promotes vasorelaxation through different mechanisms. It increases eNOS phosphorylation and NO production,[36] inhibits Ca^{2+} channels, releases Ca^{2+} from intracellular stores, and activates K^+ channels.[37] Meanwhile, amentoflavone relaxes vascular smooth muscle via the nitric oxide-cGMP pathway as well as involving non-specific K^+ and Ca^{2+} channels.[38] The vasorelaxant effect of GCE might be attributed to the combined action of the kaempferol, isovitexin, quercetin, apigenin, luteolin, and amentoflavone found in the extract.

The extraction yield is influenced by various factors, such as the solvent with varying polarity, pH level, temperature, extraction duration, and sample composition.[39] Moreover, various environmental factors such as light, temperature, soil moisture, soil fertility, and salinity, have a significant impact on secondary metabolite accumulation in plants. A change in any of these factors may alter the content of secondary metabolites, even if other factors remain consistent.[40] Therefore, several factors could contribute to the differences between our results and those of the previous study, Yorsin et al.[16] Variations in extraction methods, solvents, and the collection locations of plant material can result in different phytochemical constituents. This variation in the phytochemical composition indicates that while there might be some overlapping mechanisms of action on vasorelaxation of *G. cowa* extract because of the shared presence of kaempferol and isovitexin, there are also differences in mechanisms of action due to the presence of quercetin, apigenin, luteolin, and amentoflavone, as well as the absence of vitexin. Additional studies are needed to isolate and characterize the bioactive compounds responsible for the vasorelaxant activity of GCE.

CONCLUSION

The ethanolic extract from the leaves of *G. cowa* induced relaxation in rat thoracic aortic rings through both endothelium-dependent mechanisms, mediated by the eNOS/NO/sGC pathway, and endothelium-independent pathways involve the opening of K_{Ca} and K_V channels and inhibition of extracellular Ca^{2+} influx. Kaempferol, isovitexin, quercetin, apigenin, luteolin, and amentoflavone may contribute to the vasorelaxation effect of GCE. Additional research is necessary to isolate and identify the specific bioactive components responsible for the vasorelaxant properties of GCE.

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ABBREVIATIONS

4-AP	4-aminopyridine
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine phosphate
CGRP	Calcitonin gene-related peptide
COX	Cyclooxygenase
DMSO	Dimethyl sulfoxide
EC_{50}	The concentration of GCE that induced 50% of maximal relaxation
E_{max}	The maximal relaxation
eNOS	Endothelial nitric oxide synthase
GCE	*Garcinia cowa* ethanol extract
GPCR	$G\alpha_s$-protein-coupled receptor
K_{ATP}	ATP-sensitive K^+ channel
K_{Ca}	Calcium-activated K^+ channel
K_V	Voltage-dependent K^+ channel
L-NAME	$N^{(\omega)}$-nitro-L-arginine methyl ester
NO	Nitric oxide
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PGI_2	Prostacyclin
sGC	Soluble guanylyl cyclase
TEA	Tetraethylammonium
VOCC	Voltage-operated Ca^{2+} channels
VSMCs	Vascular smooth muscle cells

REFERENCES

1. Sriyatep T, Siridechakorn I, Maneerat W, Pansanit A, Ritthiwigrom T, Andersen RJ. Bioactive prenylated xanthones from the young fruits and flowers of *Garcinia cowa*. J Nat Prod. 2015;78(2):256-71. https://doi.org/10.1021/np5008476
2. Mahabusarakam W, Chairerk P, Taylor WC. Xanthones from *Garcinia cowa* Roxb. latex. Phytochemistry. 2005;66(10):1148-53. https://doi.org/10.1016/j.phytochem.2005.02.025
3. Panthong K, Hutadilok-Towatana N, Panthong A. Cowaxanthone F, a new tetraoxygenated xanthone, and other anti-inflammatory and antioxidant compounds from *Garcinia cowa*. Can J Chem. 2009;87(11):1636-40. https://doi.org/10.1139/V09-123
4. Murakami A, Jiwajiinda S, Koshimizu K, Ohigashi H. Screening for in vitro anti-tumor promoting activities of edible plants from Thailand. Cancer Lett. 1995;95(1-2):137-46. https://doi.org/10.1016/0304-3835(95)03879-2
5. Jantan I, Jumuddin FA, Saputri FC, Rahman K. Inhibitory effects of the extracts of *Garcinia* species on human low-density lipoprotein peroxidation and platelet aggregation in relation to their total phenolic contents. J Med Plant Res. 2011;5(13):2699-709.
6. Negi PS, Jayaprakasha GK, Jena BS. Evaluation of antioxidant and antimutagenic activities of the extracts from the fruit rinds of *Garcinia cowa*. Int J Food Prop. 2010;13(6):1256-65. https://doi.org/10.1080/10942910903050383
7. Bulbul-A-Munira, Chowdhury SA. Antibacterial, neuropharmacological and analgesic activities of *Garcinia cowa* (family: Clusiaceae). Int Conf Bioinform Biomed Eng. 2015;1(2):112-7.
8. Ritthiwigrom T, Laphookhieo S, Pyne SG. Chemical constituents and biological activities of *Garcinia cowa* Roxb. Maejo Int J Sci 2013;7(2):212-31.
9. Xu G, Kan WLT, Zhou Y, Song J, Han Q, Qiao C, et al. Cytotoxicity acylphloroglucinol derivatives from the twigs of *Garcinia cowa*. J Nat Prod. 2010;73(2):104-8. https://doi.org/10.1021/np9004147

10. Cheenpracha S, Phakhodee W, Ritthiwigrom T, Prawat U, Laphookhieo S. A new depsidone from the twigs of *Garcinia cowa*. Heterocycles. 2011;83(5):1139-44.
11. Wahyuni FS, Shaari K, Stanslas J, Lajis N, Hamidi D. Cytotoxic compounds from the leaves of *Garcinia cowa* Roxb, J Appl Pharm Sci. 2015;5(2):6-11.
12. Panthong K, Pongcharoen W, Phongpaichit S, Taylor WC. Tetraoxygenated xanthones from the fruits of *Garcinia cowa*. Phytochemistry. 2006;67(10):999-1004. https://doi.org/10.1016/j.phytochem.2006.02.027
13. Sakunpak A, Panichayupakaranant P. Antibacterial activity of Thai edible plants against gastrointestinal pathogenic bacteria and isolation of a new broad spectrum antibacterial polyisoprenylated benzophenone, chamuangone. Food Chem. 2012;130(4):826-31. https://doi.org/10.1016/j.foodchem.2011.07.088
14. Likhitwitayawuid K, Phadungcharoen T, Krungkrai J. Antimalarial xanthones from *Garcinia cowa*. Planta Med. 1998;64(1):70-2.
15. Raksat A, Phukhatmuen P, Yang J, Maneerat W, Charoensup R, Andersen RJ, et al. Phloroglucinol benzophenones and xanthones from the leaves of *Garcinia cowa* and their nitric oxide production and α-glucosidase inhibitory activities. J Nat Prod. 2020;83(1):164-8. https://doi.org/10.1021/acs.jnatprod.9b00849
16. Yorsin S, Sriwiriyajan S, Chongsa W. Vasorelaxing effect of *Garcinia cowa* leaf extract in rat thoracic aorta and its underlying mechanisms. J Tradit Complement Med. 2023;13(5):219-25. https://doi.org/10.1016/j.jtcme.2022.12.001
17. Dirar AI, Alsaadi DHM, Wada M, Mohamed MA, Watanabe T, Devkota HP. Effects of extraction solvents on total phenolic and flavonoid contents and biological activities of extracts from Sudanese medicinal plants. S Afr J Bot. 2019;120:261-7. https://doi.org/10.1016/j.sajb.2018.07.003
18. Verma N, Shukla S. Impact of various factors responsible for fluctuation in plant secondary metabolites, J Appl Res Med Aromat Plants. 2015;2(4):105-13. https://doi.org/10.1016/j.jarmap.2015.09.002\
19. Qu Z, Zhang J, Gao W, Chen H, Guo H, Wang T. Vasorelaxant effects of Cerebralcare Granule® are mediated by NO/cGMP pathway, potassium channel opening and calcium channel blockade in isolated rat thoracic aorta. J Ethnopharmacol. 2014;155(1):572-9. https://doi.org/10.1016/j.jep.2014.05.062
20. Aekthammarat D, Tangsucharit P, Pannangpetch P, Sriwantana T, Sibmooth N. *Moringa oleifera* leaf extract enhances endothelial nitric oxide production leading to relaxation of resistance artery and lowering of arterial blood pressure. Biomed Pharmacother. 2020;130:110605. https://doi.org/10.1016/j.biopha.2020.110605
21. Stankevicius E, Kevelaitis E, Vainorius E, Simonses U Role of nitric oxide and other endothelium-derived factors. Medicina (Kaunas). 2003;39(4):333-41.
22. Loh YC, Tan CS, Ch'ng YS, Yeap ZQ, Ng CH, Yam MF. Overview of the microenvironment of vasculature in vascular tone regulation. Int J Mol Sci. 2018;19(1):120. https://doi.org/10.3390/ijms19010120
23. Ch'ng YS, Loh YC, Tan CS, Ahmad M, Asmawi MZ, Omar WMW, et al. Vasorelaxant properties of *Vernonia amygdalina* ethanol extract and its possible mechanism. Pharm Biol. 2017;55(1):2083-94. https://doi.org/10.1080/13880209.2017.1357735
24. Liggett SB. Molecular and genetic basis of β_2-adrenergic receptor function. J Allergy Clin Immunol. 1999;104(2):S42-6. https://doi.org/10.1016/S0091-6749(99)70272-1
25. Panthiya L, Pantan R, Tocharus J, Nakaew A, Suksamrarn A, Tocharus C. Endothelium-dependent and endothelium-independent vasorelaxant effects of tiliacorinine 12′-*O*-acetate and mechanisms on isolated rat aorta. Biomed Pharmacother. 2019;109:2090-9. https://doi.org/10.1016/j.biopha.2018.11.062
26. Ko EA, Han J, Jung ID, Park WS. Physiological roles of K^+ channels in vascular smooth muscle cells. J Smooth Muscle Res. 2008;44(2):65-81. https://doi.org/10.1540/jsmr.44.65
27. Nelson MT, Quayle JM. Physiological roles and properties of potassium channels in arterial smooth muscle. Am J Physiol. 1995;268(4):799-822. https://doi.org/10.1152/ajpcell.1995.268.4.C799
28. Jackson WF. Ion channels and vascular tone. Hypertension. 2000;35(1):173-8. https://doi.org/10.1161/01.HYP.35.1.173
29. Calderone V, Chericoni S, Martinelli C, Testai L, Nardi A, Morelli I, et al. Vasorelaxing effects of flavonoids: investigation on the possible involvement of potassium channels. Naunyn Schmiedebergs Arch Pharmacol. 2004;370(4):290-8. https://doi.org/10.1007/s00210-004-0964-z
30. Sun YH, Zhao J, Jin HT, Cao Y, Ming T, Zhang LL, et al. Vasorelaxant effects of the extracts and some flavonoids from the buds of *Coreopsis tinctoria*. Pharm Biol. 2013;51(9):1158-64. https://doi.org/10.3109/13880209.2013.782320
31. Roghani M, Baluchnejadmojarad T, Vaez-Mahdavi MR, Roghani-Dehkordi F. Mechanisms underlying quercetin-induced vasorelaxation in aorta of subchronic diabetic rats: an in vitro study. Vascul Pharmacol. 2004;42(1):31-5. https://doi.org/10.1016/j.vph.2004.12.001
32. Khoo NK, White CR, Pozzo-Miller L, Zhou F, Constance C, Inoue T, et al. Dietary flavonoid quercetin stimulates vasorelaxation in aortic vessels. Free Radic Biol Med. 2010;49(3):339-47. https://doi.org/10.1016/j.freeradbiomed.2010.04.022
33. Hou X, Liu Y, Niu L, Cui L, Zhang M. Enhancement of voltage-gated K^+ channels and depression of voltage-gated Ca^{2+} channels are involved in quercetin-induced vasorelaxation in rat coronary artery. Planta Med. 2014;80(6):465-72.
34. Sui H, Zhi Y, Liu H, Gao P, Xu H, Yan W. Endothelium-dependent vasorelaxation effects induced by apigenin on the thoracic aorta of rats and its possible mechanism. Wei Sheng Yan Jiu. 2011;40(4):416-9.
35. Ma X, He D, Ru X, Chen Y, Cai Y, Bruce IC, et al. Apigenin, a plant-derived flavone, activates transient receptor potential vanilloid 4 cation channel. Br J Pharmacol. 2012;166(1):349-58. https://doi.org/10.1111/j.1476-5381.2011.01767.x
36. Si H, Wyeth RP, Liu D. The flavonoid luteolin induces nitric oxide production and arterial relaxation. Eur J Nutr. 2014;53(1):269-75. https://doi.org/10.1007/s00394-013-0525-7
37. Jiang H, Xia Q, Wang X, Song J, Bruce IC. Luteolin induces vasorelaxion in rat thoracic aorta via calcium and potassium channels. Pharmazie. 2005;60(6):444-7.
38. Kang DG, Yin MH, Oh H, Lee DH, Lee HS. Vasorelaxation by amentoflavone isolated from *Selaginella tamariscina*. Planta Med. 2004;70(8):718-22. https://doi.org/10.1055/s-2004-827201
39. Do QD, Angkawijaya AE, Tran-Nguyen PL, Huynh LH, Soetaredjo FE, Ismadji S, et al. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. J Food Drug Anal. 2014;22(3):296-302. https://doi.org/10.1016/j.jfda.2013.11.001
40. Yang L, Wen K-S, Ruan X, Zhao Y-X, Wei F, Wang Q. Response of plant secondary metabolites to environmental factors. Molecules. 2018;23(4):762. https://doi.org/10.3390/molecules2304076

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