# กลไกการคลายตัวของหลอดเลือดโดยเคอซิตินสกัดจาก *อะนาซาโกเรีย ลูโซเนนซิส* เอ เกรย์ ในเอออร์ตาของหนูแรท

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**บทคัดย่อ**

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลการคลายตัวของหลอดเลือดเอออร์ตาของหนูแรทโดย quercetin ที่สกัดจาก *Anaxagorea luzonensis* รวมทั้งกลไกที่เกี่ยวข้อง ผลการศึกษาพบว่า quercetin ที่ความเข้มข้น 1-100 μM มีฤทธิ์ทำให้เกิดการคลายตัวของหลอดเลือดแบบ concentration-dependent ซึ่งผลนี้ถูกยับยั้งเมื่อชั้นเยื่อบุหลอดเลือดถูกทำลาย และโดย $N^G$-nitro-L-arginine methyl ester (L-NAME) ที่ความเข้มข้น 300 μM แต่ indomethacin ที่ความเข้มข้น 10 μM ไม่มีผลต่อฤทธิ์ของ quercetin การเพิ่มขึ้นของ KCl ภายนอกเซลล์ที่ความเข้มข้น 60 mM สามารถยับยั้งการตอบสนองของหลอดเลือดโดย quercetin นอกจากนี้ การคลายตัวของหลอดเลือดโดย quercetin ยังถูกยับยั้งโดย tetraethyammonium ที่ความเข้มข้น 5 mM, barium chloride ที่ความเข้มข้น 30 μM และ glibenclamide ที่ความเข้มข้น 10 μM แต่ไม่ถูกยับยั้งโดย 4-aminopyridine ที่ความเข้มข้น 1 mM เมื่อนำหลอดเลือดมาแช่ด้วย quercetin ที่ความเข้มข้น 1-100 μM มีผลยับยั้งการหดตัวของหลอดเลือดโดยแคลเซียมคลอไรด์และ methoxamine ผลการศึกษานี้โดยใช้หลอดเลือดเอออร์ตาของหนูแรทแสดงให้เห็นว่า การตอบสนองของหลอดเลือดต่อ quercetin มีกลไกบางส่วนเกี่ยวข้องกับไนตริกออกไซด์ นอกจากนี้ ยังเกี่ยวข้องกับช่องทางผ่านของโพแทสเซียมไอออนชนิด $K_{IR}$, $K_{Ca}$, $K_{ATP}$ ผลการศึกษาที่น่าสนใจคือ ฤทธิ์ของ quercetin ส่วนใหญ่เกิดจากการยับยั้งการเข้าสู่เซลล์ของแคลเซียมไอออนจากภายนอกเซลล์

**คำสำคัญ:** *อะนาซาโกเรีย ลูโซเนนซิส* เคอซิติน การคลายตัวของหลอดเลือดแดง ชั้นเยื่อบุหลอดเลือด ช่องทางผ่านของโพแทสเซียมไอออน การเข้าสู่เซลล์ของแคลเซียมไอออน

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# Mechanisms of vasorelaxation induced by Quercetin from *Anaxagorea luzonensis* A. Grey in the rat aorta

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## Abstract

The aim of the present study was to study the vasorelaxant effects of quercetin from *Anaxagorea luzonensis* and its underlying mechanisms in the aortas of rats. Quercetin (1-100 μM) induced concentration-dependent vasorelaxations were reduced by endothelial denudation, and 300 μM *NG*-nitro-L-arginine methyl ester (L-NAME), but not 10 μM indomethacin. After raising the extracellular KCl concentration to 60 mM inhibited vasorelaxant responses to quercetin. Moreover, the responses to quercetin were inhibited by 5 mM tetraethyammonium, 30 μM barium chloride, and 10 μM glibenclamide, but not 1 mM 4-aminopyridine. Pre-incubation with quercetin (1-100 μM) inhibited the contractions induced by CaCl2 and methoxamine. The present findings demonstrated that in the rat isolated aorta, that vasorelaxant responses to quercetin were mediated in part via Nitric Oxide dependent pathways. Moreover, the activation of KIR, K Ca, K ATP channels seemed to play a role in quercetin-induced responses. Interestingly, the inhibition of extracellular Ca2+ influx is largely involved in the action of quercetin.

**Keywords:** *Anaxagorea luzonensis,* quercetin, vasorelaxation, endothelium, K+ channels, Ca2+ influx

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## Introduction

The heartwood of *Anaxagorea luzonensis* A. Grey (AL), belonging to Annonacea family, has been widely used in Thai traditional medicine as a health promoting herb, blood tonic, antihistamine, and antihypertensive agents.[1,2] However, there are less scientific bases for its pharmacological effects. A previous study has reported that methanolic extract of AL, containing flavones, flavonones, flavonols, exerts an estrogenic activity.[3] Moreover, some xanthones and flavonoids found in methanolic extract of heartwood of AL have antioxidant activity.[2] A recent study has demonstrated that dichloromethane extract of AL ($CH_2Cl_2$-AL) induces vasorelaxation which is partly mediated via the endothelium-dependent pathway. Moreover, activation of $K^+$ channels and inhibition of extracellular $Ca^{2+}$ influx involve the vasorelaxant effects of $CH_2Cl_2$-AL.[4] However, there is no evidence of vascular effect of quercetin, extracted and purified from AL.

## Objectives

The present study aimed to investigate the effect of the quercetin on vascular tone and its underlying mechanisms in the isolated rat aorta.

## Materials and methods

### Extraction of quercetin from *Anaxagorea luzonensis* A. Grey (AL)

The isolation of quercetin from *A. luzonensis* was previously reported.[5,6] Briefly, the dried and chopped heartwood (5 kg) of AL was extracted four times with methanol using soxhlet extractor. After filtration, the methanolic extract was concentrated under reduced pressure and was then partitioned with dichloromethane ($CH_2Cl_2$), yielding about 10.5 g of $CH_2Cl_2$-AL after evaporation. Then, quercetin was extracted and purified from $CH_2Cl_2$-AL by column chromatography silica gel. Molecular weight of quercetin was 302.236.

### Tissue preparation

Experiments were performed using aorta obtained from male Wistar rats (300 - 350 g). Rats were housed in standard environmental condition (25°C) under 12 h light/dark cycle and fed with standard laboratory rat chow and tap water *ad libitum*. All experimental procedures were reviewed and approved by the Animal Research Ethics Committee of the Faculty of Medicine, Srinakharinwirot University.

Male Wistar rats were anaesthetized with Zolitil 50 mg/kg (tiletamine chloridrate and zolazepan chloridrate) into quadriceps muscle, and killed by cervical dislocation. Following a thoracotomy, the thoracic aorta was carefully removed, cleaned of fat and connective tissue and cut into 5 mm ring segments. Each ring was transferred to a jacketed organ bath filled with 20 mL of modified Krebs-Henseleit solution, composed of (mM) NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, D-glucose 10 that was maintained at 37°C, and bubbled continuously with 95% $O_2$ and 5% $CO_2$ mixture.

The solution in the organ bath was exchanged every 15 minutes for 1 h. The rings were mounted between two triangular stainless-steel hooks that were passed through the lumen and stretched to an optimal passive tension of about 1 g, and maintained at this tension for 1 h. The upper hook was connected to isometric force transducer (MLT 0210, New South Wales, Australia), and changes in isometric force were recorded on a MacLab recording system (AD instruments, New South Wales, Australia).

**Experimental protocol**

Following a 1-hour equilibration period, methoxamine (10-60 μM) was used to increase tone by approximately 1 g. Once a stable contraction was established, quercetin (1-100 μM) was added cumulatively. To explore the mechanisms involved in vasorelaxation induced by quercetin, aortic rings were incubated with various inhibitors added to the organ bath before methoxamine was added to increase tone. In vehicle-control experiments, dimethyl sulphoxide (DMSO) alone was added cumulatively in the same volumes as those used in the experiments with quercetin.

To examine the contribution of the endothelium in vasorelaxant responses to quercetin, the endothelium was mechanically removed by gently rubbing the luminal surface with a cocktail stick. Removal of the endothelium was demonstrated by vasorelaxation to 10 μM carbachol being less than 10% of the induced tone. To investigate the involvement of vasodilator prostanoids via the cyclooxygenase (COX) pathway and nitric oxide (NO) in vasorelaxation to quercetin, aortic rings were treated with indomethacin (10 μM), a COX inhibitor, and $N^G$-nitro-L-arginine methyl ester (L-NAME, 300 μM), an inhibitor of endothelial nitric oxide synthase, respectively.

To investigate the potential involvement of $K^+$ channels in vasorelaxation to quercetin, aortic rings were pre-contracted with a high extracellular concentration of KCl (60 mM), which was prepared by replacing an equimolar concentration of NaCl with KCl.[7] These experiments were also performed in endothelium-denuded rings to determine any involvement of endothelium-derived relaxing factors (EDRFs) in the vasorelaxant effects of quercetin on activation of $K^+$ channels. To characterized the types of $K^+$ channels involved in vasorelaxation to quercetin, concentration-response curves to quercetin were constructed after incubation with tetraethylammonium (TEA, 5 mM), a non-specific $K^+$ channel inhibitor, 4-aminopyridine (4-AP, 1 mM), a voltage-gated $K^+$ ($K_V$) channel inhibitor, glibenclamide (10 μM), an ATP-sensitive ($K_{ATP}$) inhibitor, or barium chloride ($BaCl_2$, 30 μM), an inward-rectifier ($K_{IR}$) channel inhibitor.

To examine the vascular effect of quercetin on extracellular $Ca^{2+}$ influx, concentration-response curve to $CaCl_2$ (10 μM-30 mM) were constructed in the presence and absence of quercetin (1, 10 and 100 μM) for 30 minutes. Aortic rings were first

allowed to equilibrate at 1 g tension in a $Ca^{2+}$-free Krebs solution, and then the rings were bathed with $Ca^{2+}$-free, high KCl (100 mM) Krebs solution. In vehicle-control experiments, DMSO was added in the same volume as that used in the experiments with quercetin. In addition, to examine the effects of quercetin on contractile responses of aortic rings to vasoconstrictors, aortic rings were preincubated with quercetin (1, 10 and 100 μM) for 30 minutes before concentration-response curves of methoxamine (0.1 - 300 μM), an adrenergic receptor agonist.

**Data and statistical analysis**

The concentration of vasorelaxant giving half-maximal relaxation ($EC_{50}$) and maximal responses ($R_{max}$) were obtained from the concentration-response curve fitted to a sigmoidal logistic equation using the GraphPad Prism package.[7] $R_{max}$ and $pEC_{50}$ values (negative logarithm of the $EC_{50}$) were compared by analysis of variance (ANOVA) with statistically significant differences between groups being determined by Bonferroni's post-hoc test. These were expressed as mean±SEM. The results were considered statistically significant when *p* value was less than 0.05. The number of animals in each group is represented by n.

**Chemicals**

All drugs and chemicals were purchased from Sigma Chemical Company (St. Louis, Missouri, USA), but zoletil was purchased from Virbac (Carros Cedex, France). Indomethacin was dissolved in ethanol. Quercetin and glibenclamide were dissolved in DMSO. 4-AP and $BaCl_2$ were dissolved in distilled water. The remaining drugs were dissolved in the Krebs solution. All drugs were made up on the day of the experiment.

**Results**

**The effects of endothelial denudation, indomethacin and L-NAME on vasorelaxation to quercetin in rat aortic rings**

Quercetin (1-100 μM) caused vasorelaxation in a concentration-dependent manner (Figure 1). Endothelium denudation significantly ($p<0.001$) reduced vasorelaxation to 100 μM quercetin (control = 74.1±6.9%; denuded = 22.2±3.6%, n = 6, Figure 1). Similarly, vascular responses to 100 μM quercetin were significantly ($p<0.01$) reduced by 300 μM L-NAME (control = 74.1±6.9%; L-NAME = 36.5±4.6%, n = 6, Figure 1). However, indomethacin did not affect vasorelaxant responses to quercetin (Figure 1).

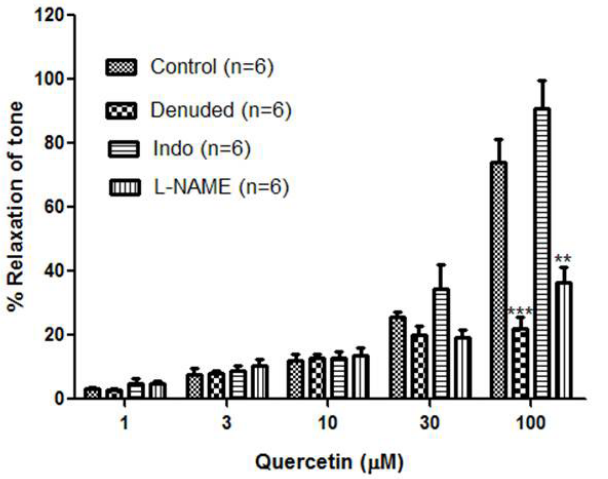


**Figure 1** The effects of endothelial denudation, indomethacin (10 μM), and $N^G$-nitro-L-arginine methyl ester (L-NAME, 300 μM) on quercetin-induced vasorelaxation in rat aortic rings. Data are shown as mean ± SEM.

**The effects of high extracellular potassium and potassium channel inhibitors on vasorelaxation to quercetin**

Raising extracellular $K^+$ concentration (60 mM KCl) significantly ($p$<0.001) inhibited vasorelaxation induced by 100 μM quercetin (control = 74.1±6.9%; 60 mM KCl = 21.6±5.2%, n=6, Figure 2). Similarly, the responses to 100 μM quercetin was significantly ($p$<0.001) inhibited by 5mM tetraethylammonium (control = 74.1±6.9 %; 60 mM KCl = 17.2±2.3%, n = 6, Figure 2). Moreover, relaxant responses to quercetin were significantly reduced by either barium cholride or glibenclamide, but not 4-AP ($BaCl_2$ = 45.7±1.8%, $p$<0.05, n=6; glibenclamide = 40.3±4.4%, $p$<0.01, n=6, Figure 3).

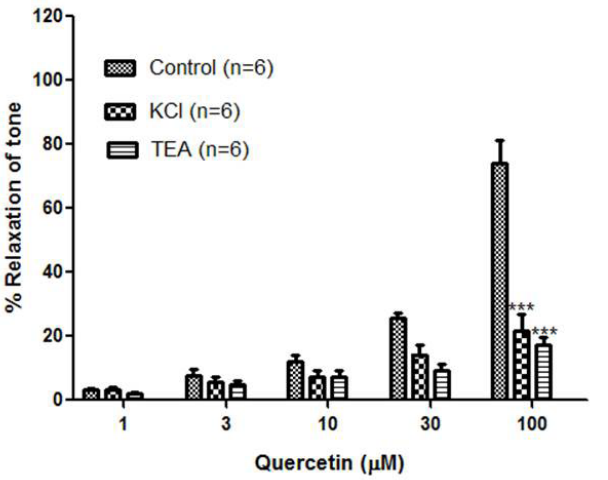


**Figure 2** The effects of 60 mM KCl and tetraethylammonium (TEA, 5mM) on quercetin-induced vasorelaxation in rat aortic rings. Data are shown as mean ± SEM.

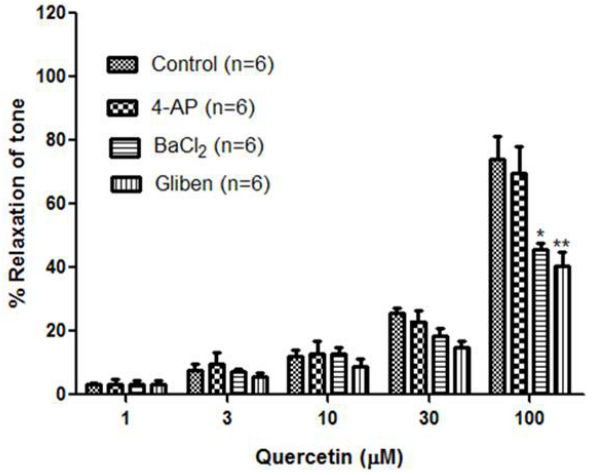


**Figure 3** The effects of 4-aminopyridine (4-AP, 1 mM) or barium chloride ($BaCl_2$, 10 μM) glibenclamide (Glib, 10 μM) (B) on quercetin-induced vasorelaxation in rat aortic rings. Data are shown as mean ± SEM.

**The effects of quercetin on extracellular $Ca^{2+}$ influx in rat aortic rings**

$CaCl_2$ (10 μM-30 mM) elicited concentration-dependent contraction of KCl (100 mM) depolarized rings in $Ca^{2+}$-free medium. Contractions to $CaCl_2$ were significantly reduced by quercetin in a concentration-dependent manner ($R_{max}$: control = 0.92±0.03 g, n=6; 10 μM quercetin = 0.76±0.04 g, $p$<0.01, n=6; 100 μM quercetin = 0.15±0.01 g, $p$<0.01, n=6, Figure 4).

Pre-incubation with quercetin (1, 10 and 100 μM) significantly inhibited maximal contractions to methoxamine ($R_{max}$: control = 0.95±0.05 g, n=5; 1 μM quercetin = 0.79±0.04 g, $p<0.05$, n=5; 10 μM quercetin = 0.29±0.05 g, $p<0.01$, n=5; 100 μM quercetin = 0.05±0.01 g, $p<0.001$, n=5; Figure 5).

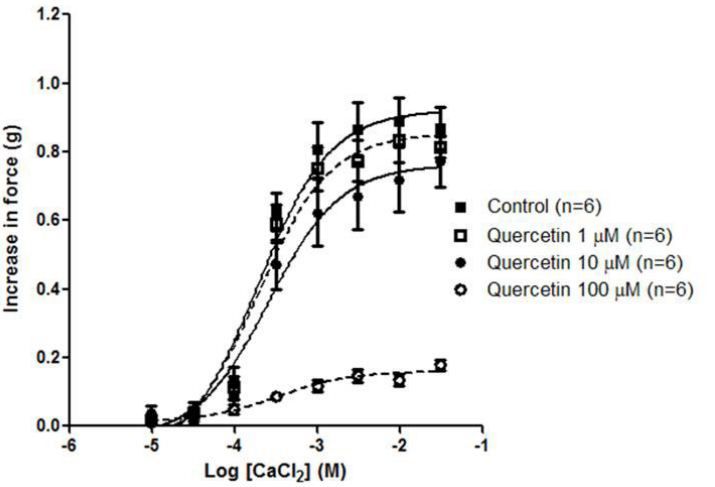


**Figure 4** The effects of quercetin (1, 10 and 100 μg/ml) on $CaCl_2$-induced contraction in rat aortic rings depolarized by 100 mM KCl. Data are shown as mean ± SEM.

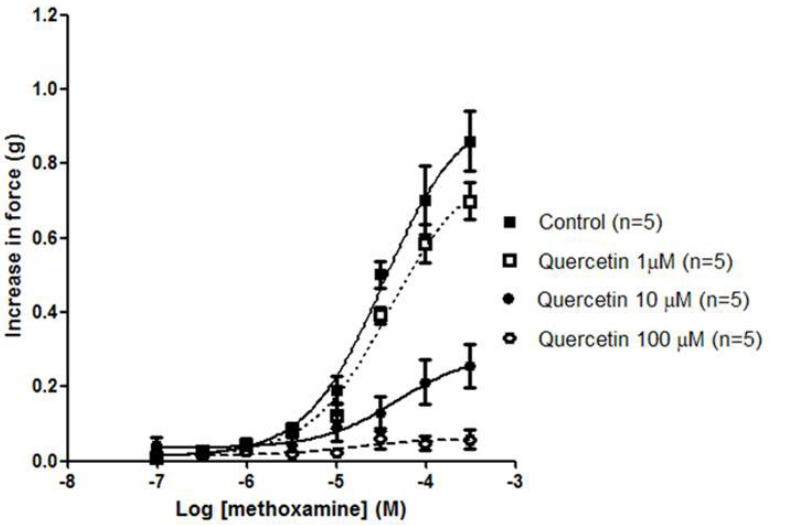


**Figure 5** The effects of quercetin (1, 10 and 100 μg/ml) on methoxamine-induced contraction in rat aortic rings depolarized by 100 mM KCl. Data are shown as mean ± SEM.

## Discussion

The present study has demonstrated the vasorelaxant effects of quercetin and mechanisms involved in its action. Quercetin causes vasorelaxation in the isolated rat aorta, which is partly due to endothelium-derived NO. Activation of $K^+$ channels and inhibition of $Ca^{2+}$ influx are largely involved in the vasorelaxant effects of quercetin.

Previous studies have reported that the extract of *Anaxagorea luzonensis* A. Gray contains several flavones and flavonoids.[2,3] Our previous study has shown that $CH_2Cl_2$-AL causes vasorelaxation in rat aortic rings.[4] The present findings support that quercetin is involved in vasorelaxant responses to $CH_2Cl_2$-AL.

The vascular endothelium plays an essential role in regulating vascular tone via synthesis and release endothelium-derived relaxing factors (EDRFs), such as NO and prostacyclin.[8,9] The present experiments show that relaxations induced by quercetin were inhibited by removal of the endothelium. Moreover, the effects of quercetin were inhibited by L-NAME, a NOS inhibitor, but not inhibition of COX pathway by indomethacin. These results indicate the participation of endothelium-derived NO in the effects of quercetin. Therefore, these results indicate that the relaxant responses to quercetin involve NO, but not vasodilator prostanoids via the COX pathway.

Several types of $K^+$ channels are located in vascular smooth muscle cells, including $K_{ATP}$, $K_{Ca}$, $K_V$, and $K_{IR}$ channels. Opening of $K^+$ channels in the cell membrane of vascular smooth muscle cells increases $K^+$ efflux, causing hyperpolarization, which closure of voltage-gated $Ca^{2+}$ channels and subsequently vasorelaxation.[10,11] To investigate the involvement of $K^+$ channels in vasorelaxant effects of quercetin, a high concentration of KCl was used to increase vascular tone. It was found that 60 mM KCl inhibited the effects of quercetin. These findings suggest that quercetin causes vasorelaxation directly by increasing $K^+$ efflux through $K^+$ channels on smooth muscle cells.

In order to investigate the contribution of exact types of $K^+$ channels involved in vasorelaxant effects of quercetin, we used different types of $K^+$ channel inhibitors. It was found that vasorelaxation induced by quercetin were inhibited by tetraethylammonium (TEA, 5 mM), a non-specific $K^+$ channel inhibitor, barium chloride, a $K_{IR}$ channel inhibitor, glibenclamide, a $K_{ATP}$ channel inhibitor. Conversely, 4-aminopyridine, a $K_V$ channel inhibitor, did not affect vascular responses to quercetin. These results suggest that vasorelaxant responses to quercetin are mediated by increasing $K^+$ efflux, at least in part, through $K_{IR}$, $K_{ATP}$, $K_{Ca}$ channels.

Another aspect examined in the present study was whether vasorelaxant responses to inhibition of extracellular $Ca^{2+}$ influx involved. We found that contractile responses of rat aortic rings to $CaCl_2$ in $Ca^{2+}$-free medium containing KCl were inhibited by

quercetin. $\alpha_1$-Adrenoreceptor agonists, such as methoxamine, cause vasoconstriction via activation of protein kinase C to increase extracellular $Ca^{2+}$ influx through receptor-operated $Ca^{2+}$ channels, and/or $Ca^{2+}$ release from intracellular store.[12,13] We found that quercetin inhibit methoxamine-induced vasoconstriction. Taken together, these findings support the notion that quercetin can block $Ca^{2+}$ influx from the extracellular space.

## Conclusions

Our findings have demonstrated that quercetin exerts its vasorelaxant effects by acting on multiple sites of actions in the rat aorta. Vascular responses to quercetin are partly mediated through endothelium-dependent NO. Additionally, inhibition of extracellular $Ca^{2+}$ entry and activation of $K^+$ channels are required for the vasorelaxant effects of quercetin.


## Acknowledgments

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