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Relaxatsion Responses To Extracts of Polygonum Hydropiper (L) In Porcine Coronary Artery: Role of Potassium Channel

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ABSTRACT

Polygonum hydropiper (L) is commonly known as "smart weed" that is used traditionally for the management of hypertension. Therefore, the aim of this study was to determine the effect of *P. hydropiper* extracts on vasorelaxation using porcine coronary artery rings.Segments of porcine coronary artery were mounted for isometric tension recording in isolated tissue baths and pre-contracted with the thromboxane A2 analog U46619.After pre-contraction, cumulative concentrations of *P. hydropiper* extracts were added to the tissues to determine the fraction with greatest activity. A variety of inhibitors of intracellular signaling pathways were then utilized in order to determine the mechanism of relaxation. *P. hydropiper* extracts produced a concentration dependent relaxation of the porcine coronary artery, with the butanol soluble fraction producing the greatest response. The relaxation to the butanol soluble extract was not reduced by removal of endothelium indicating endothelium-independent mechanism of relaxation. Furthermore, the relaxation was unaffected by removal of extracellular calcium, and pre-incubation with the extract had no effect on contractions due to influx of extracellular calcium or release of intracellular calcium. However, relaxation responses were decreased in the presence of potassium channel blockers. Relaxation responses to sodium nitroprusside and forskolin were enhanced by the presence of the butanol soluble extract, suggesting phosphodiesterase inhibitory action. Indeed, relaxation responses to the PDE 4 and PDE 5 inhibitors rolipram and sildenafil were similarly inhibited by potassium channel blockers, indicating that the effect of the butanol extract on potassium channels may be related to phosphodiesterase activity. Theobromine and gallic acid identified as constituents of butanol soluble fraction of *P. hydropiper*, also produced a relaxation that was inhibited by potassium channel blockers, suggesting that the vascular activity of butanol soluble fraction of *P. hydropiper* may be due to the presence of theses compound. In conclusion, butanol soluble fraction of *P. hydropiper* produces relaxation in porcine coronary artery, which is dependent upon opening of potassium channel, potentially downstream of phosphodiesterase inhibition.

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Introduction

Vasoconstriction is one of the most common risk factor for cardiovascular diseases such as coronary artery disease and hypertension.[1]Vasoconstriction occurs by contraction of the vascular smooth muscle cells through both calcium dependent and calcium-sensitization pathways.[2] Vascular tone is a balance between vasodilatory signals and vasoconstriction. Endothelium-derived vasodilators such as nitric oxide and prostacyclin cause smooth muscle relaxation through increases in cGMP and cAMP respectively, leading to inhibition of contractile pathways.[3] A number of plants have been used traditionally in the treatment of cardiovascular diseases, but for the most part the scientific basis of these effects is unknown.

Polygonum hydropiper (L.) belongs to the family Polygonaceae [4] and synonyms for this species is *Persicariahydropiper* (L.). It grows in large amounts in the northern areas of Pakistan, where it is commonly known as "smart weed".[5] It is used traditionally for its reported antihypertensive and diuretic activity[6], as well as other indications, including pain, inflammation, flatulence, and aiding digestion.[7] Previously studies have reported its pharmacological effects as antioxidant, anti-nociceptive, neuropharmacological[8], anthelminthic[9] and anti-Alzheimer's effects.[10] Phytochemical analysis of *P. hydropiper* has identified the presence of flavonoids and polyphenols such as sesquiterpenes, sesquiterpenoids, and phenylpropanoids, (+)-catechin, (−)-epicatechin, hyperin, isoquercitrin isorhamnetin kaempferol, quercetin and gallic acid.[5]In spite of its traditional use for treatment of cardiovascular disease, the effects of *Polygonum hydropiper* on vascular tone has not yet been determined. Therefore, the aim of this study was to determine the effect of a crude extract of *Polygonum hydropiper* (L) on vascular tone in the porcine coronary artery. Different extracts of *Polygonum hydropiper* (L) were then determined for bioactivity, and the mechanism of action of the extract with the greatest activity investigated.

Material and methods

Plant Material

The aerial parts of *Polygonum hydropiper* was harvested from the Gilgit area of Pakistan during the beginning of rainyseason in August, 2017. The plant was taken to the Departmentof Botany, Govt. College University Faisalabad (GCUF) Faisalabad-Pakistan for authentication. A voucher sample (W-1064) of the plantwas deposited in the herbarium of Department of Pharmacy, Faculty of Pharmaceutical Sciences, Govt. College University Faisalabad-Pakistan.

Preparation of *Polygonum hydropiper* crude extract/fractions

The aerial parts of plant of *Polygonum hydropiper* (12 kg) were dried in fresh air under shade for 3 days and pulverizedinto coarse powder. Thecoarse plant material was subsequently extractedwith 70% of aqueous methanol solution and kept for 3 days. The resulting crude methanolic extract was filtered bypassage through a Whatmann no. 1 filter paper and repeated procedure three times, followed byconcentration at 40◦C using a rotary evaporatorand freeze drying. The yield of the freeze-dried sample representing aqueous methanolic extract of *P. hydropiper* (AMPH) was calculated to be 31.35%.[11] Forthe preparation of fractions, 250g of the extract was suspended in 250mL deionized waterand mixed thoroughly. The mixture was transferred toa separating funnel for sequential fractionation by solvent-solvent extraction method with sequential additionof ethylacetate (3×100 mL), and n-butanol (3×100 mL). The resulting fractions collectedinto separate conical flasks were concentrated in rotary evaporator and subsequently freeze-dried to obtain ethyl acetate soluble fraction (13.01g), butanol soluble fraction (24.21g), aqueous soluble fraction (61.50g). We did not obtained any quantity of hexane and dichloromethane soluble fractions. Crude extract and fractions were stored in a refrigerator at -4°C, until used for more pharmacological investigation.[12]

Animals

Fresh hearts of adult pigs of either sex (~40-50 kg) were collected from a local abattoir and transported to the laboratory in ice-cold Krebs'-Henseleit solution.

Drugs

Chemicals used for the preparation of Krebs-Henseleit buffer solution were purchase from Fisher Scientific, UK. Caffeine, TEA, 4-aminopyridine, barium chloride, glibenclamide, gallic acid, sodium nitroprusside (SNP), EGTA and theobromine were purchased from Sigma-Aldrich, UK. U46619, BayK8644, ionomycin, cyclopiazonic acid (CPA), forskolin, ODQ, PMA, Y27632, apamin, TRAM-34, iberiotoxin, rolipram, sildenafil were obtained from Tocris Biosciences (Bristol, UK). All the chemicals of standard analytical grade.

LC/Q-TOF-MS analysis of butanol soluble fraction of *P. hydropiper*

LC-MS/Q-TOF-MS analysis was performed by Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source. For the chromatographic separation, a column of (Agilent Zorbax Esclipse XDB-C18, Narrow-Bore 2.1×150mm, 3.5micron) was used and two solvents, solvent A (0.1 percent formic acid with water) and solvent B (0.1 percent formic acid with acetonitrile) were collected by mobile phase. The flow rate was 0.5ml / min and injection volume was 1 µl. For analysis of MS parameters, ion polarity of positive and negative ion mode used. The temperature of the drying gas (N2) was 300oC, at a gas flow rate of 10L/min, and a nebulizing pressure (N2) of 45 psig. Samples were prepared into 1mg/ml concentration and filtered with 0.22uM Nylon syringe filter and diluted with 10X with methanol before analysis.[13]

Effect of *P. hydropiper* extracts on porcine coronary artery

For this purpose, hearts (40-50g) from freshly slaughtered pigs were transported from a local abattoir to the laboratory in ice-cold Krebs–Henseleit buffer solution. From each heart, the anterior descending coronary artery was dissected and refrigerated overnight at 4°C in Krebs–Henseleit solution (composed, in mM, of the following: NaCl, 128; KCl, 4.8; $MgSO_4$, 1.1; $NaHCO_3$, 25; $KH2PO_4$, 1.2; D-glucose, 12; $CaCl_2$, 1.25. and pre-gassed with oxygen- carbon dioxide mixture (95:5). The following day, arteries were cleaned of all adipose and connective tissue and cut into 4 mm ring segments. Tissues were set up in 5 ml isolated tissue baths containing Krebs–Henseleit solution gassed with 95% O_2, 5% CO_2 and maintained at 37°C and tension measured with an isometric force transducer connected to AD Instruments Power lab. Changes in tension were recorded using LabCart version 8 (ADIntruments). A

tension of 8g tension was applied to each ring segment following a 20 min equilibration period.

After equilibration period, the tissues were exposed two times to 60 mM KCl to determine their maximal contractile capacities, with thorough rinsing and a 15-20-min recovery period following each KCl challenge. Finally, the tissues were pre-contracted to approximately 65–80% of the maximal KCl contractile response using the thromboxane mimetic U46619 (concentration range 1nM–10nM). Increasing, cumulative additions of extracts of *P. hydropiper* (3ug to 900ug/ml dissolved in distilled water) when then added.[14]

In another set of experiments, the endothelium removed by gentle rubbing of forceps in the rings before setting up in the isolated tissue baths. The removal of the endothelium was confirmed by a loss in the relaxation response to substance P (100nM) after U46619 contraction.

Effect of pre-contraction with KCl on the relaxation response.

Tissues were contracted with a sub-maximal concentration of KCl (30mM) or U46619 as a comparison. Concentration response curves to *P. hydropiper* extracts were then carried out.

Effect of extracellular calcium on relaxation response.

After second KCl contraction, treated tissue were incubated with calcium-free Krebs-Henseleit buffer containing EGTA (2mM) for 20 min before contraction with U46619. Control rings tissues were incubated with normal Krebs-Henseleit buffer. The concentration of U46619 added was adjusted to ensure a similar level of tone in calcium-containing and calcium-free Krebs-Henseleit buffer. Cumulative concentration response curves to the *P. hydropiper* extracts (3ug/ml to 900ug/ml) were then carried out. In a separate set of experiments, tissues were exposed to a single concentration of the butanol fraction of *P. hydropiper*(900 µg/ml) in calcium free Krebs-Henseleit solution without EGTA. After 45 minutes, 60mM KCl was added a concentration response curve constructed with cumulative addition of calcium chloride (1µM to 10mM). In another experiment, tissues were pre-incubated with to a single concentration (900ug/ml) of the butanol fraction of *P. hydropiper* before concentration response curves to the calcium channel opener BAY K8644 (1nM to 3uM) were carried out.

Effect of intracellular calcium on relaxation response to BSH

The tissues were incubated with/without fraction (900ug/ml) in calcium containing Kreb solution. Then after 45 min a single concentration of caffeine (10mM-ryanodine receptor activator) was added and produced contraction was recorded. In another sets of experiments, investigated that effect of relaxation is due to blocking of release of calcium from membrane pores or Ca^{2+} ATPase. For this, tissues were incubated with or without a single concentration of extract in Krebs-Henseleit buffer. After 45 minutes, a single concentration of ionomycin (10µM- calcium ionophore), or cyclopiazonic acid (CPA-10uM), a Ca^{2+} ATPase inhibitor, was added and contractile responses attained measured for 60 minutes. In a further set of experiments, the PCA segments were pre-incubated with or without a single concentration (900ug/ml) of extract for 45 minutes prior to the addition of PMA (100 µM), an activator of protein kinase C (PKC) and contractile responses measured for 60 minutes.

Evaluation of role of inhibition of Rho kinase in vasorelaxation

In order to determine whether the butanol fraction of *Polygonum hydropiper* (3ug/ml to 900ug/ml) might act through inhibition of Rho kinase, tissues were incubated with the Rho Kinase inhibitor Y27632 (10µM) for 45 minutes before pre-contraction to U46619. The concentration of U46619 was adjusted in order to obtain the same level of pre-contraction in control tissues and those treated with Y27632.

Effect of potassium channel blockers on relaxation response.

Segments of PCA were incubated with TEA (non-selective K^+ channel blocker, 10mM), 4-aminopyridine (voltage gated potassium channel blocker (K_V); 4AP; 1mM), barium chloride (inwardly rectifying potassium channel blocker (K_{IR}) (30µM), glibenclamide (K_{ATP} channel blocker; 1µM), iberiotoxin (BK_{ca} blocker (100nM), TRAM-34 (IK_{ca} blocker, 10 µM) or apamin (SK_{ca} blocker (0.5 µM) for 45 minutes prior to pre-contraction with U46619. Cumulative concentration response curves to the butanol fraction of *P.hydropiper* were then performed.

Evaluation of the role of soluble guanylyl cyclase in the relaxation response.

Segments of PCA were incubated with the soluble guanylyl cyclase inhibitor, ODQ (10 µM) for 45 minutes before contraction with U44619. Cumulative concentration response curves to the butanol fraction of *P. hydropiper* were then performed.

Effect of *P. hydropiper* extract onrelaxation responses to cyclic nucleotides.

In order to determine the effect of *P. hydropiper* extract oncAMP and cGMP-dependent relaxation responses, tissues were pre-incubated with extract (300µg/ml) for 45 minutes before pre-contraction with U46619. Cumulative concentration response curves to forskolin (1nM to 1µM) or sodium nitroprusside (SNP; 1nM to 1µM) were then carried out.

Effect of calcium activated potassium channel blockers in relaxation responses to phosphodiesterase inhibitors

The butanol fraction of *P. hydropiper* enhanced the relaxation responses to SNP and forskolin, suggesting it may act as a phosphodiesterase inhibitor. As the calcium-activated potassium channel blockers inhibited the relaxation to the *P. hydropiper* extract, we determined whether known inhibitors of PDE4 (cAMP selective PDE isoform) or PDE5 (cGMP selective isoform) could produce a relaxation of the PCA which was also sensitive to inhibition by potassium channel blockers. Segments

of PCA were pre-incubated with iberiotoxin (BK_{ca} blocker-100nM), TRAM-34 (IK_{ca} blocker -10µM) and apamin (SK_{ca} 0.5µM) before contraction with U46619.Cumulative concentration response curves to rolipram (PDE4 inhibitor) or sildenafil (PDE5 inhibitor) were then performed.

Determination of vasorelaxant effect of theobromine on porcine coronary artery

LC-MS analysis of butanol soluble fraction of *P. hydropiper* identified the presence of theobromine, a known inhibitor of phosphodiesterase. Therefore, in order to determine whether theobromine could also produce a relaxation which was sensitive to inhibition of calcium-activated potassium channels, segments of PCA were pre-incubated with iberiotoxin (BK_{ca} blocker-100nM), TRAM-34 (IK_{ca} blocker 10µM) and apamin (SK_{ca} 0.5µM) before contraction with U46619.Cumulative concentration response curves to theobromine (10^{-6} M to 10^{-1}M) were performed.

Data analysis

Relaxations were expressed as a percentage relaxation from the U46619 or KCl-induced pre-contraction. Contractile responses were expressed as a percentage of the contraction to 60mM KCl. Responses were expressed mean ± SEM of n experiments, where n indicates the number of different animals used. Concentration-response data were analyzed using a 2-way ANOVA, followed by a Bonferroni post-hoc test. Responses to single concentrations of drugs were analyzed using a Student's, 2-tailed, paired t-test. $P<0.05$ was considered statistically significant.

Results

LC/Q-TOF-MS analysis of butanol soluble fraction of *P. hydropiper*

LC-MS analysis of butanol soluble fraction of *P. hydropiper* was executed and obtained constituents were given in Table 1. Phytochemical analysis exposed the presence of more than 10 compounds with prominent theobromine, gallic acid and gingerol respectively.

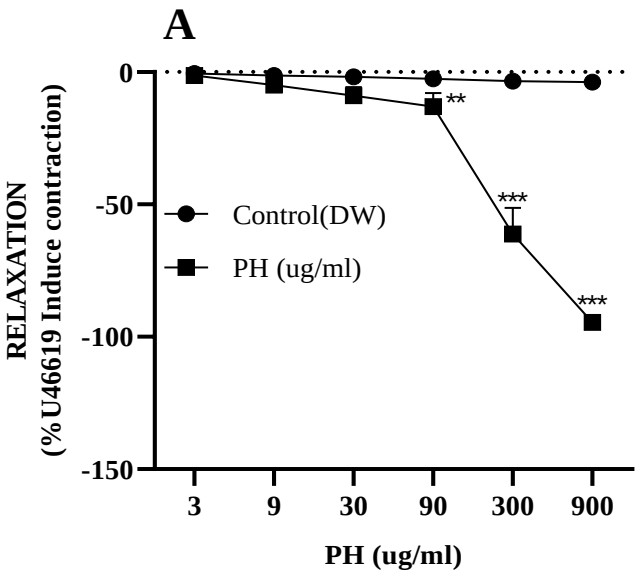
Table1: LC-MS analysis of butanol soluble fraction of *Polygonum hydropiper*

Sr.no	Compound name	R.T	m/z	DB Diff (ppm)	Molecular formula	Mass
1	Theobromine	0.647	179.0581	-4.11	C7 H8 N4 O2	180.0655
2	Hypoxanthine	0.65	135.0311	0.89	C5 H4 N4 O	136.0384
3	2,4-Imidazolidinedione, 5-(7-oxabicyclo[4.1.0]hepta-2,4-dien-3-yl)-5-phenyl-	0.651	267.0764	4.29	C15 H12 N2 O3	268.0836
4	L-Lyxose	0.68	149.046	-2.6	C5 H10 O5	150.0532
5	3,3-Dimethyl-1,2-dithiolane	0.729	133.0154	-2.52	C5 H10 S2	134.0227
6	Fumaric acid	0.73	115.0043	-5.56	C4 H4 O4	116.0116
7	Chlorpropamide	0.736	275.0253	2.78	C10 H13 Cl N2 O3 S	276.0328
8	N-Acryloylglycine	0.92	128.0357	1.96	C5 H7 N O3	129.0423
9	Erythrono-1,4-lactone	1.085	117.0199	-4.66	C4 H6 O4	118.0272
10	Gallic acid	1.549	169.014	1.58	C7 H6 O5	170.0213

11	6-Hydroxyluteolin 3'-methyl ether 7-sulfate	9.431	395.0082	-0.89	C16 H12 O10 S	396.0155
12	(6S)-dehydrovomifoliol	12.326	221.1192	-3.75	C13 H18 O3	222.1264
13	Gingerol	13.143	293.1759	0.1	C17 H26 O4	294.1831

P. hydropiper **extract/fraction vasorelaxation effect on endothelium intact/denuded porcine coronary artery rings**

The aqueous methanolic extract of *P. hydropiper* (AMPH) produced concentration dependent vasorelaxation in endothelium intact ring pre-contracted with U46619 as compared to vehicle control (Fig.1A). Moreover, all subsequent fractions of this extract produced vasorelaxation, but the butanol soluble fraction produced the greatest relaxation, which was similar to the aqueous methanolic extract of *P. hydropiper* (control) (Fig: 1B). As this fraction had the largest bioactivity, the mechanism underlying the relaxation to this fraction was investigated further. Removal of the endothelium had no effect on the relaxation response to the butanol soluble fraction compared to intact rings (Fig. 2A).



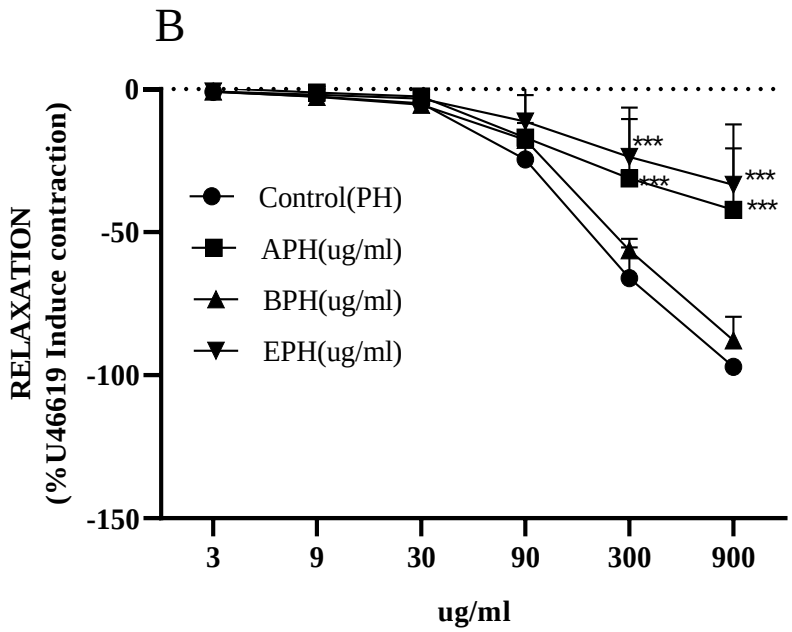
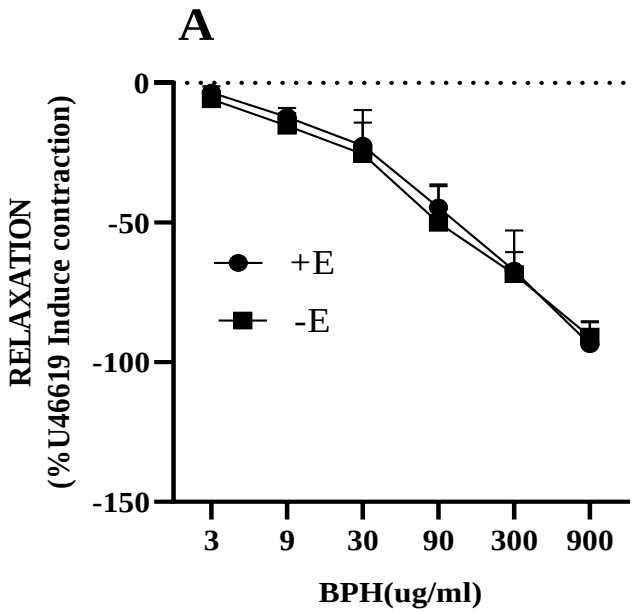


Figure:1 Vasorelaxant effect of aqueous methanolic extract of *Polygonum hydropiper*(AMPH) in porcine coronary artery ring precontracted with U46619, compared with distilled water (DW) as control (A),compared with ethyl acetate fraction (EPH), butanol fraction (BPH) and aqueous fraction (APH) (B).Data are expressed as a percentage relaxation from the U46619-induced tone and are means ± SEM of (n=6), **** indicate $p<0.0001$***$p<0.001$, **$p<0.01$, *$p<0.05$, 2 way ANOVA followed by Bonferroni post-hoc test.

Vasorelaxation effect of *P. hydropiper* active fraction on contraction induced by KCl

The butanol soluble fraction also produced vasorelaxation in rings pre-contracted with KCl, although the relaxation at the maximum concentration used was lower than that seen after pre-contraction with U46619 (Fig. 2B).



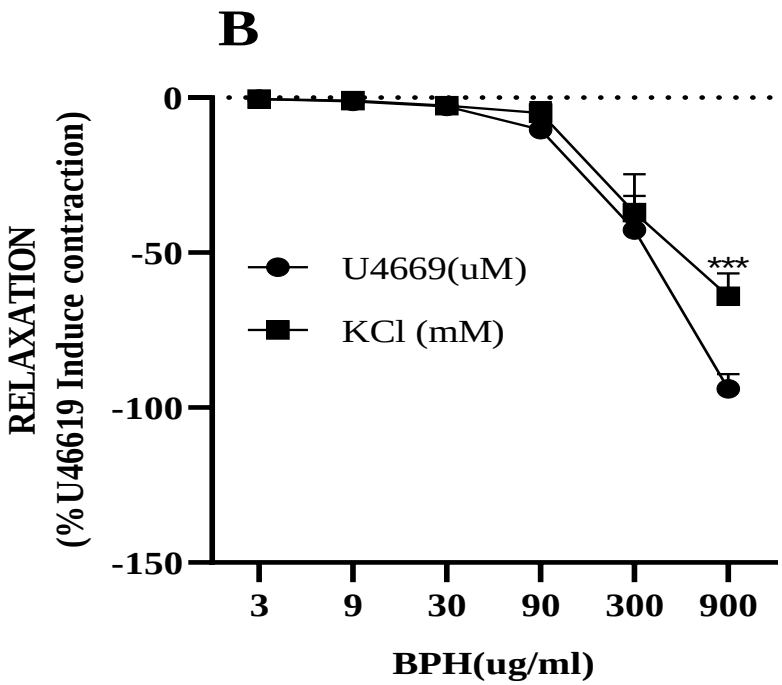
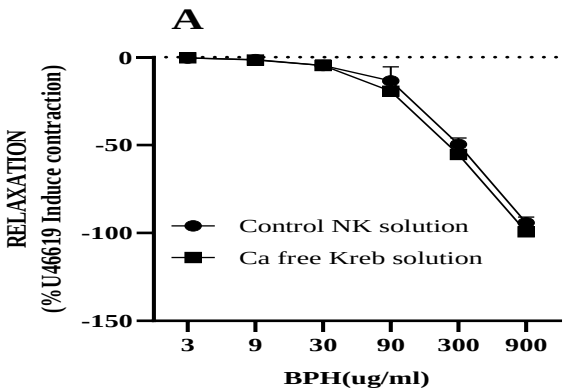
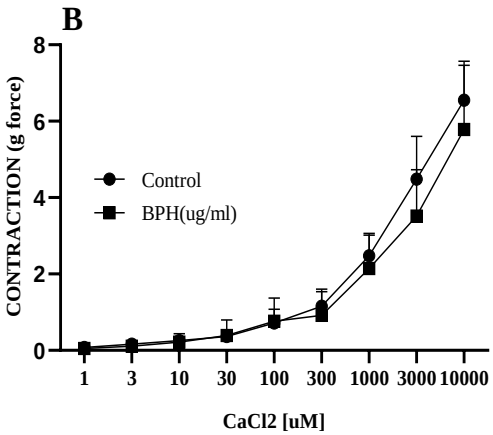


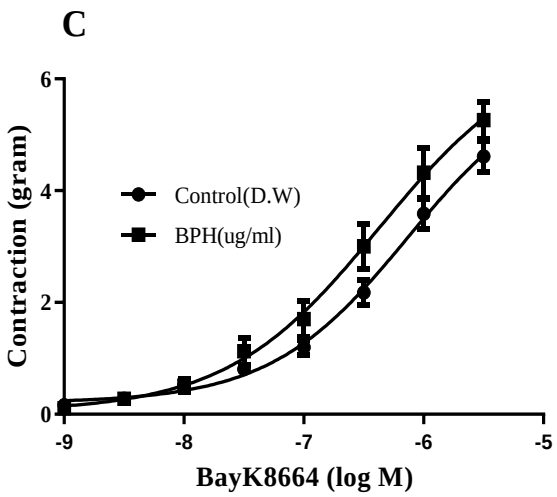
Figure:2 Vasorelaxant effect of butanol fraction of *Polygonum hydropiper* **(**BPH) in endothelium intact (E+) and endothelium denuded (E) porcine coronary artery rings pre-contracted with U46619 (A), Vasorelaxant effect of butanol fraction of *Polygonum hydropiper*(BPH) in porcine coronary artery rings pre-contracted with KCl compared with U46619 (B).Data are expressed as a percentage relaxation from the U46619-induced tone and are means ± SEM of (n=6), *** indicate $p<0.001$, **$p<0.01$, *$p<0.05$, 2 way ANOVA followed by Bonferroni post-hoc test.

***P. hydropiper* active fraction vasorelaxation not affected by removal of extracellular calcium**

Removal of extracellular calcium did not affect the relaxation to the butanol soluble fraction (Fig.3A). Furthermore, pre-incubation with 900µg/ml of the butanol soluble fraction did not alter the contraction in response to addition of calcium in porcine coronary rings stimulated with KCl (60mM) in absence of extracellular calcium (Figure: 3B). Also, this fraction did not prevent the contraction to the L-type calcium channel opener BAY K8644 (Fig. 3C).




B
CONTRACTION (g force)
Control
BPH(ug/ml)
CaCl2 [uM]


C
Contraction (gram)
Control(D.W)
BPH(ug/ml)
BayK8664 (log M)

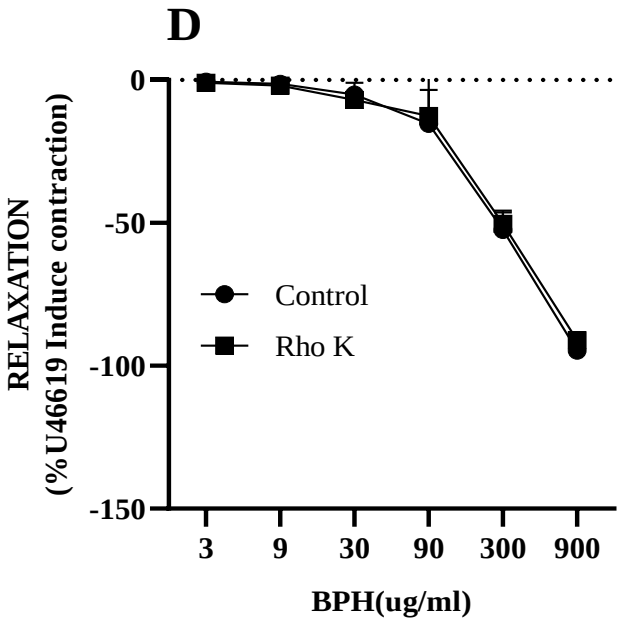

D
RELAXATION
(%U46619 Induce contraction)
Control
Rho K
BPH(ug/ml)

Figure: 3 Vasorelaxant effect of butanol fraction of *Polygonum hydropiper*(BPH) in porcine coronary artery rings precontracted with U46619 in presence of normal Kreb solution (NK) and Ca free Kreb solution (A). With cumulative addition of calcium chloride ($CaCl_2$=1µM to 10mM) (B), with commulative addition of BayK8644 (10^{-6} to 10^{-2} M) (C), in presence of Rho Kinase inhibitor (Y-27632 dihydrochloride =10µM) (D), as compared to distilled water (DW). Data are expressed as a percentage relaxation from the U46619-induced tone and are means ± SEM of (n=6), 2 way ANOVA followed by a Bonferoni post-hoc test.

***P. hydropiper* active fraction vasorelaxation is not prevented by Rho kinase inhibitor**

As there was no effect on calcium-induced contractions, we determined whether the butanol soluble fraction might affect calcium-sensitization pathways such as Rho kinase. Therefore, tissues were pre-incubated with the Rho kinase inhibitor Y27632 (10µM) prior to contraction with U46619. Inhibition of Rho kinase had no effect on the relaxation response to the butanol soluble fraction of *P. hydropiper* compared to control (Fig. 3D).

***P. hydropiper* active fraction does not inhibit calcium-dependent contractions**

Pre-incubation with the butanol soluble fraction of *P. hydropiper* (900µg/ml) did not inhibited the contraction induced due the caffeine (Fig. 4A), CPA (Fig. 4B), ionomycin (Fig. 4C) or the phorbol ester PMA (Fig. 4D) in porcine coronary rings compared to control rings.

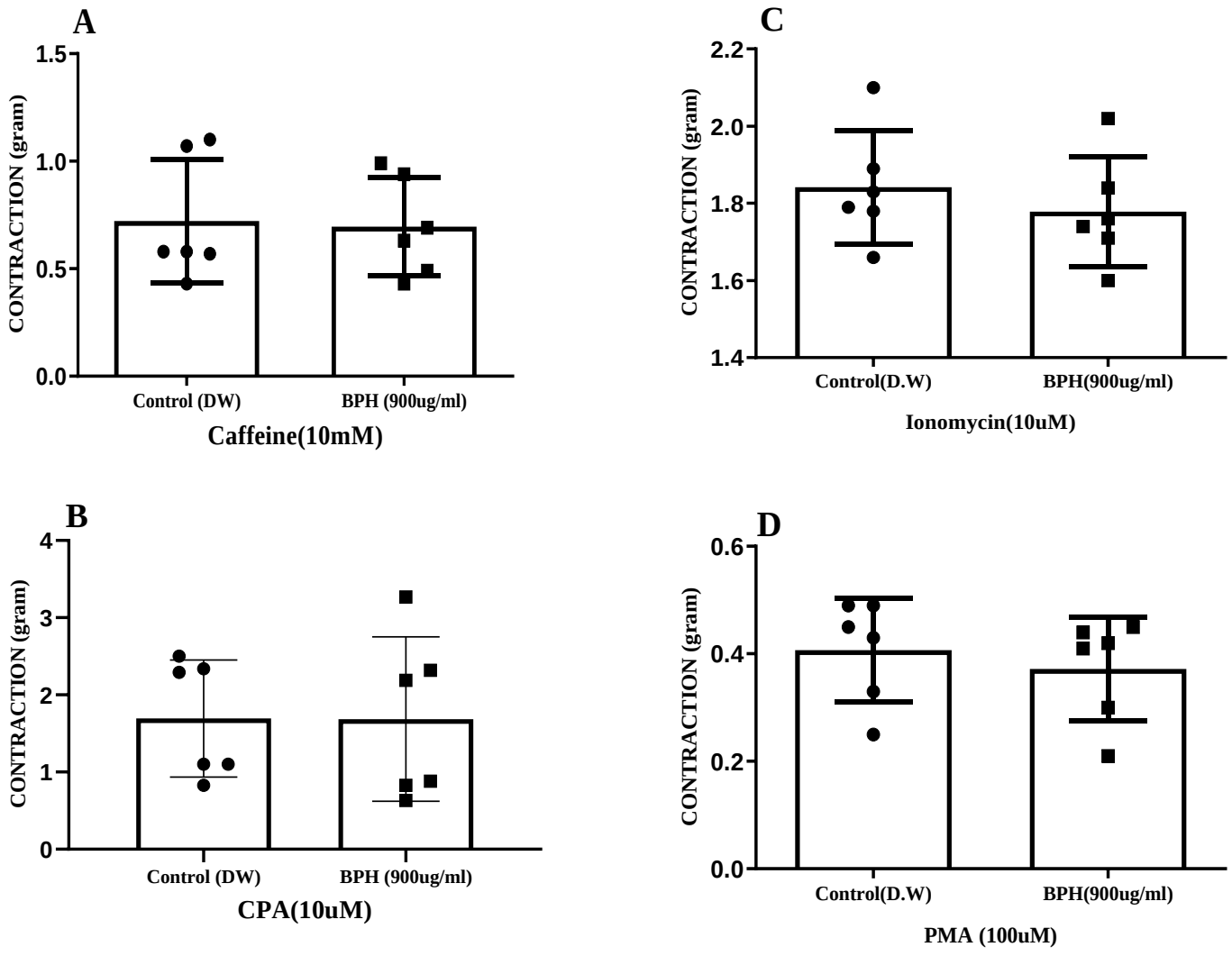


Figure: 4 Vasorelaxant effect of butanol fraction of *Polygonum hydropiper*(BPH) in porcine coronary artery rings with caffeine (A), cyclopiazonic acid (CPA=10µM) (B), ionomycin (10µM) (C), PMA (100µM) (D) compared to distilled water DW. Data are expressed as a contraction produced due to CPA and are means ± SEM of (n=6). One way ANOVA followed by a Bonferoni post-hoc test.

***P. hydropiper* active fraction vasorelaxation inhibited by potassium channel inhibitors**

Pretreatment with the non-selective potassium channel inhibitor TEA produced a slight inhibition of the relaxation to the butanol soluble fraction of *P. hydropiper* (Fig. 5A). The Kv channel inhibitor 4-AP, however, had no effect on the relaxation (Fig.5B). Pre-treatment with the K_{IR} inhibitor barium chloride, the K_{ATP} channel inhibitor glibenclamide, the BK_{ca} channel inhibitor iberiotoxin, the IK_{ca} channel inhibitor TRAM 34, and the SK_{ca} channel inhibitor apamin all produced significant inhibition of the relaxation (Fig.). Moreover, a combination of glibenclamide, iberiotoxin, TRAM- 34 and apamin appeared to inhibit the relaxation to a greater extent (~50% inhibition) (Fig 6D).

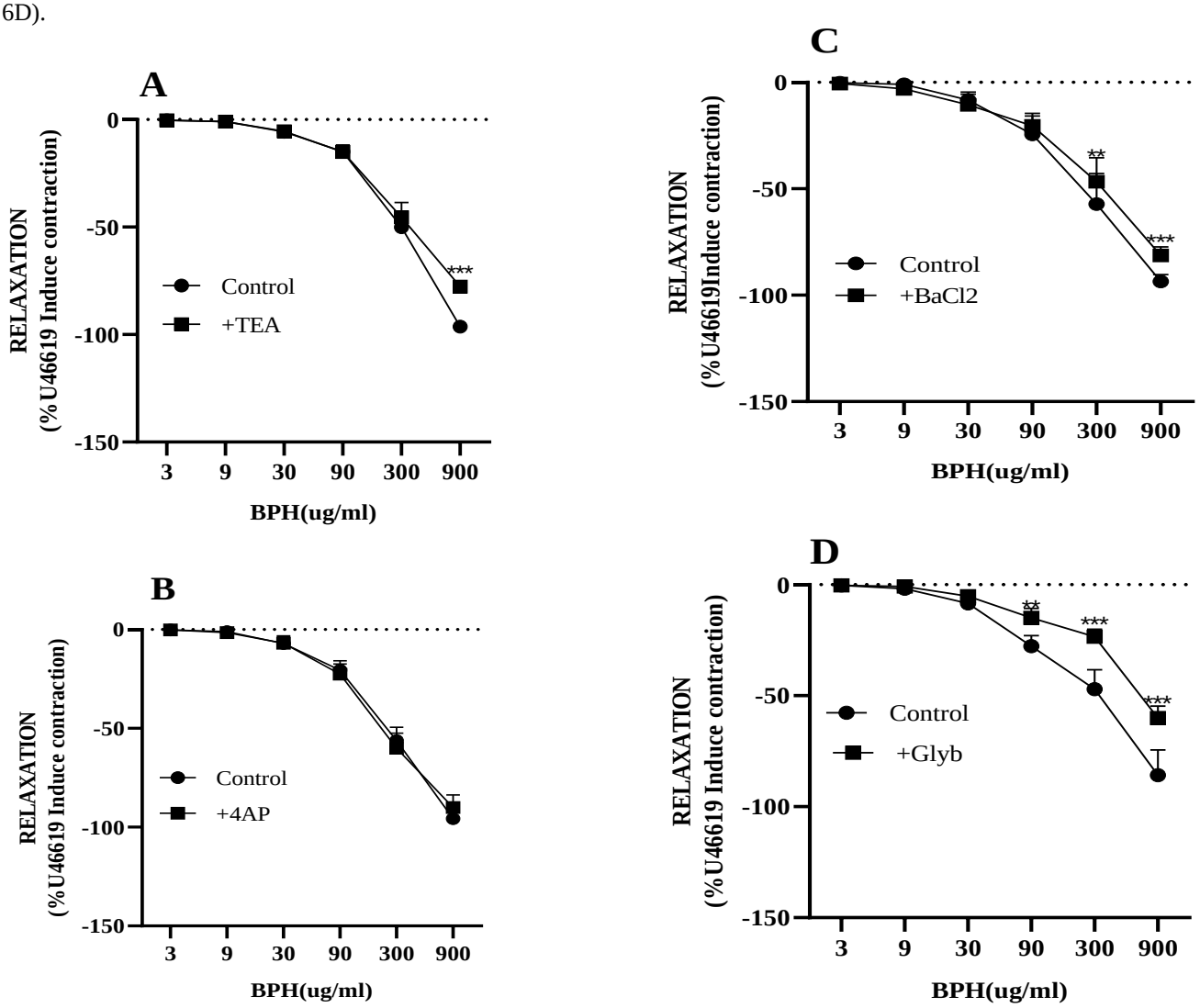


Figure: 5 Vasorelaxant effect of butanol fraction of *Polygonum hydropiper* in rat aorta rings precontracted with U44619, with absence and presence of tetraethylammonium (TEA=10mM) (**A)**, 4 aminopyridine (4AP=1mM) (**B)**, barium chloride (BaCl2=30uM)) **(C)** and glybenclamide (Glyb=1uM) (**D)**. Data are expressed as a percentage relaxation from the U46619-induced tone and are means ± SEM of (n=6), *** indicate $p<0.001$, ** $p<0.01$, * $p<0.05$, 2 way ANOVA followed by a Boneferoni post-hoc test.

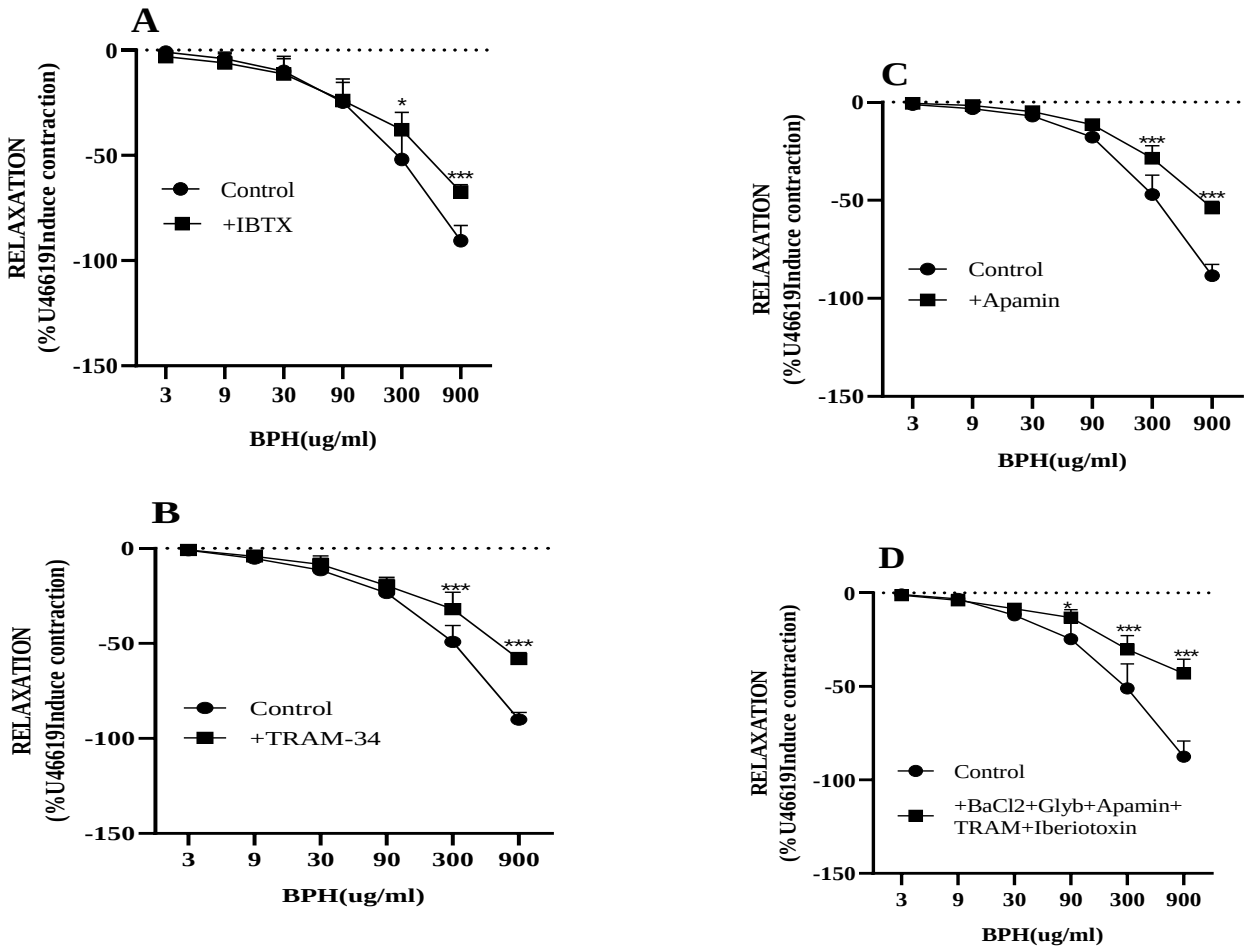


Figure: 6 Vasorelaxant effect of butanol fraction of *Polygonum hydropiper* in rat aorta rings pre-contracted with U44619, with absence and presence of iberiotoxin (Ibtx=100nM) (**A)**, TRAM-34 (10uM) (**B)**, apamin=0.5uM **(C)** and BaCl2+Glyb+apamin+TRAM+Ibtx (**D)**. Data are expressed as a percentage relaxation from the U46619-induced tone and are means ± SEM of (n=6), *** indicate $p<0.001$, ** $p<0.01$, * $p<0.05$, 2 way ANOVA followed by Bonferroni post-hoc test

Role of cGMP and cAMP pathways in relaxation response to *P. hydropiper* active fraction

Pre-incubation with the soluble guanylyl cyclase inhibitor ODQ did not affect the relaxation response to the butanol soluble fraction of *P. hydropiper* (Fig. 7). However, pre-incubation with 900µg/ml of the butanol soluble fraction of *P. hydropiper* enhanced the relaxation response to sodium nitroprusside (SNP (Fig. 8A &8B)

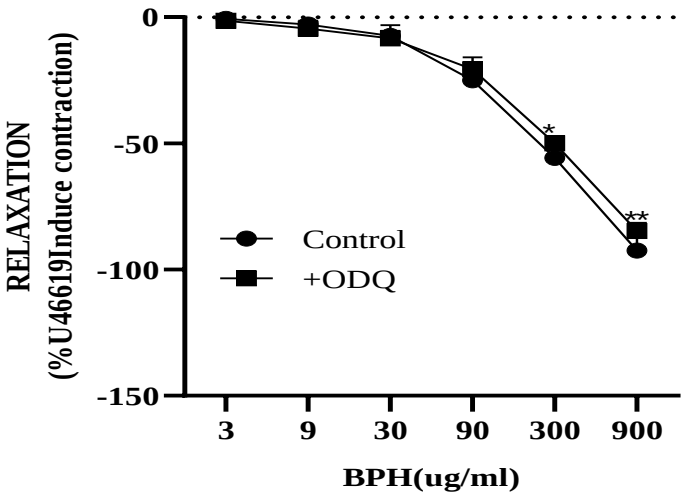


Figure: 7 Vasorelaxant effect of butanol fraction of *Polygonum hydropiper*(BPH) in porcine coronary artery rings pre-contracted with U46619, in presence of soluble guanylyl cyclase inhibitor (ODQ=10uM) compare to distilled water (DW). Data are expressed as a percentage relaxation from the U46619-induced tone and are means ± SEM of (n=6), ** indicate $p<0.01$, *$p<0.05$, 2 way ANOVA followed by a Boneferoni post-hoc test

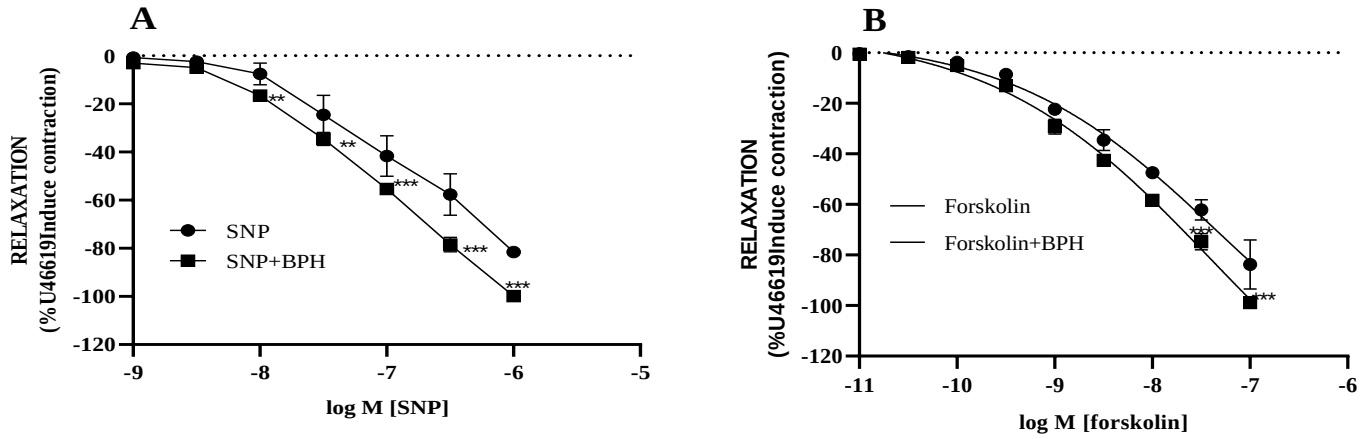


Figure: 8 Vasorelaxant effect of sodium nitroprusside (SNP=10^{-6} to 10^{-3} M) (A) and forskolin (10^{-6} to 10^{-3} M) (B), in porcine coronary artery rings pre-contracted with U46619, in absence and presence of butanol fraction of *Polygonum hydropiper*(BPH). Data are expressed as a percentage relaxation from the U46619-induced tone and are means ± SEM of (n=6), *** indicate $p<0.001$, **$p<0.01$, *$p<0.05$, 2 way ANOVA followed by Bonferroni post-hoc test

Vasorelaxation response of phosphodiesterase inhibitors inhibited by calcium activated potassium channel inhibitors

Rolipram (PDE 4 inhibitor) induced concentration-dependent relaxation of the porcine coronary artery which was inhibited by iberiotoxin and apamin (Fig. 9A).Similarly,sildenafil (PDE 5 inhibitor) also produced a concentration-dependent relaxation which was inhibited by iberiotoxin and apamin (Fig. 9B).

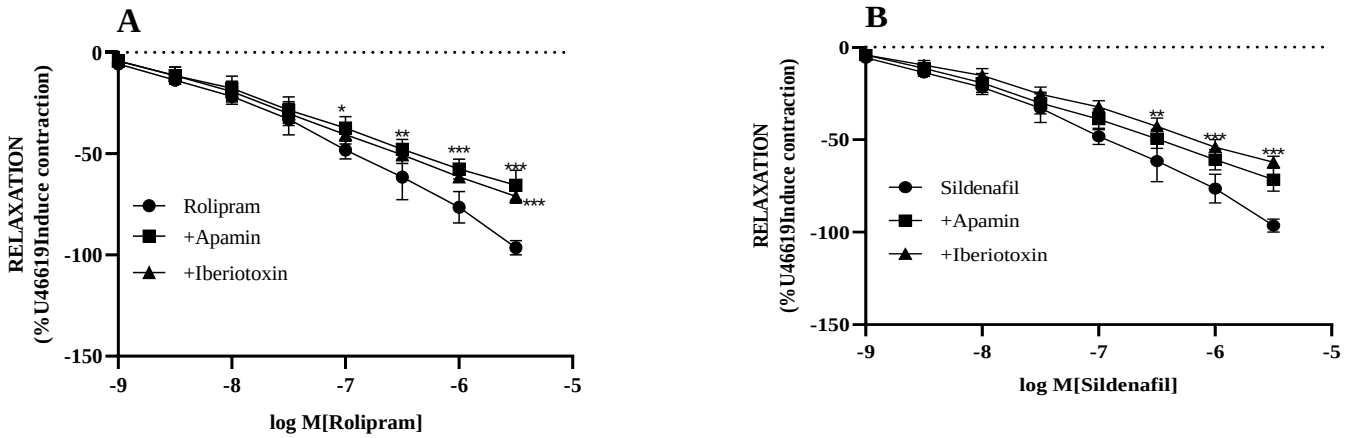
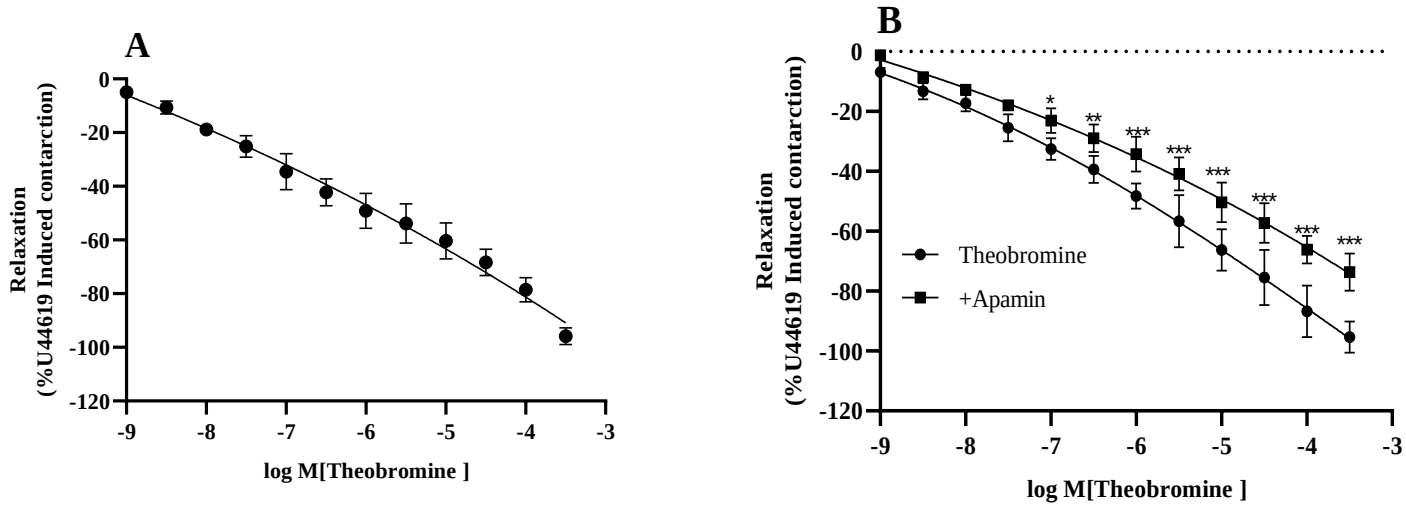


Figure: 9 Vasorelaxent effect of rolipram (PDEs 4 inhibitor) (A) and sildenafil (PDEs 5 inhibitor) (B), in rat aorta rings pre-contracted with U44619 in absence and presence of apamin (0.5uM) and iberiotoxin (100nM) (B). Data are expressed as a relaxation from the U46619-induced tone and are means ± SEM of (n=6), *** indicate $p<0.001$, ** $p<0.01$, * $p<0.05$, 2 way ANOVA followed by a Boneferoni post-hoc test

Vasorelaxant effect of theobromine in porcine coronary artery

Theobromine (a major constituent obtained by LC-MS analysis of butanol soluble fraction of *P. hydropiper*) produced a concentration- dependent relaxation ofthe porcine coronary artery, which was inhibited by pre-incubation with iberiotoxin, TRAM-34, and apamin (Fig.10) Vasorelaxation response of theobromine affected by calcium activated potassium channel blocker.



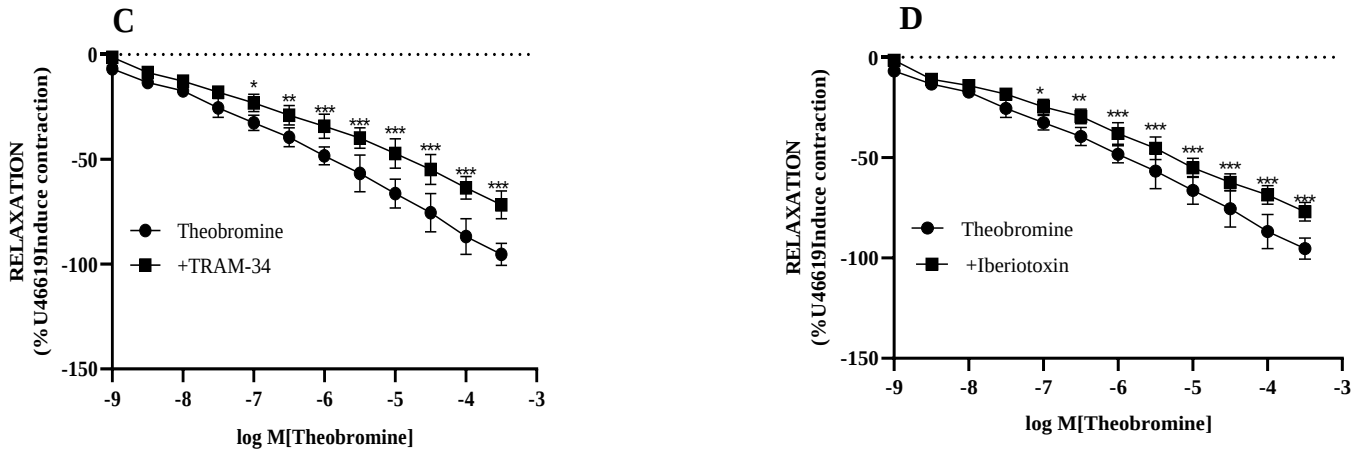


Figure: 10 Vasorelaxent effect of theobromine (10^{-6} to 10^{-1} M) in rat aorta rings pre-contracted with U44619 (A), in absence and presence of apamin (0.5uM) (B) ,TRAM-34 (10uM) and iberitoxin (100nM) (D). Data are expressed as a relaxation from the U46619-induced tone and are means ± SEM of (n=6), *** indicate $p<0.001$, **$p<0.01$, *$p<0.05$, 2 way ANOVA followed by Bonferroni post-hoc test

Discussion

Changes in vascular tone play an important role in the regulation of total peripheral vascular resistance and control of blood flow to organs. Vascular tone is regulated by vascular endothelial and smooth muscle cells and these cells are sites of action for various compounds that lower blood pressure and increase tissue perfusion.[15] These compounds can act on the endothelium to release vasoactive compounds, which subsequently affect vascular smooth muscle tone. Alternatively, they may act directly on the smooth muscle.[16] The endothelial cells release vasodilator compounds such as nitric oxide (NO), prostacyclin, and endothelium derived hyperpolarizing factor (EDH). Endothelium-independent relaxation includes activation of potassium channels, inhibition of calcium channels, increasing levels of cyclic nucleotides, and effects on calcium sensitization pathways. The data presented in this study demonstrate that extracts of *P. hydropiper* produced an endothelium-independent relaxation of the porcine coronary artery with the relaxation response to the butanol soluble fraction of *P. hydropiper* greater than other fractions, indicating the presence of bioactive compounds within this fraction. Therefore, further investigation was conducted with this fraction.

The butanol soluble fraction of *P. hydropiper* produced a relaxation response after pre-contraction with U46619 and KCl, although the relaxation after pre-contraction with KCl was reduced at the highest concentration of *P. hydropiper* extract. The contraction to both high potassium and U46619, acting through thromboxane receptors, involves influx of extracellular calcium through opening of voltage-gated calcium channels.[16] Therefore, the relaxation response to *P. hydropiper* extract could involve inhibition of calcium influx. However, pre-incubation with the butanol soluble fraction of *P. hydropiper* had no effect on contractions in response to influx of extracellular calcium. Furthermore, the contractions in response to release of intracellular calcium due to caffeine and cyclopiazonic acid were unaffected by *P. hydropiper* extract. Similarly, the contraction to the calcium ionophore ionomycin was unaffected. These data indicate that *P. hydropiper*extract does not inhibit calcium influx or inhibition of a calcium-dependent signaling pathway.

Activation of TP thromboxane receptors by U46619 can lead to contractions in the absence of extracellular calcium, suggesting that it can activate calcium-sensitization contractile pathways.[17] Contractile responses to U46619 are reduced in the presence of the Rho kinase inhibitor Y27632, indicating that Rho kinase may be involved in the calcium-sensitization contractions to U46619.[18] As the butanol soluble fraction of *P. hydropiper* produced a greater relaxation after pre-contraction with U46619, which was unaffected by removal of extracellular calcium, we hypothesized that the relaxation could be due to inhibition of this Rho kinase pathway. However, the relaxation response to the butanol soluble fraction of *P. hydropiper* was unaltered in the presence of a high concentration of the Rho kinase inhibitor Y27632, suggesting that the butanol soluble fraction of *P. hydropiper* does not act through inhibition of this signaling pathway. Furthermore, the contraction to the phorbol ester phorbol myristate acetate (PMA) was also unaffected by the butanol soluble fraction of *P. hydropiper* indicating that it does not act through inhibition of protein kinase C pathways.

High potassium can reduce relaxation responses caused by opening of plasma membrane potassium channels by altering the balance of K^+ ions across the membrane. The non-selective potassium channel inhibitor TEA caused an inhibition of the relaxation to the butanol soluble fraction of *P. hydropiper* similar to that seen in the presence of KCl, suggesting that

potassium channels may be involved in the relaxation response. Selective inhibitors of different potassium channels, including K_{ATP} and calcium-activated potassium channels, also produced a similar reduction in the relaxation response, indicating multiple potassium channels may be involved in the relaxation response. Indeed, when the potassium channel inhibitors were combined, a more substantial inhibition of the relaxation response was seen (figure 6).

Although the data indicate a role for potassium channels in the relaxation response to the butanol soluble fraction of *P. hydropiper,* there was not complete inhibition of the relaxation. This indicates that other signaling pathways may be involved. The cyclic nucleotides cGMP and cAMP produce smooth muscle relaxation. Pre-incubation with the butanol soluble fraction of *P. hydropiper* enhanced the relaxation to SNP (which stimulates the production of cGMP) and forskolin (which stimulates the production of cAMP). This suggests that *P. hydropiper* might also produce relaxation through enhancement of the cyclic nucleotide pathways, for example, by inhibition of phosphodiesterase. This could be due to the presence of theobromine, a member of the xanthine group of compounds known to inhibit phosphodiesterases. Furthermore, *P. hydropiper* contains a number of flavonoids, including quercetin [9], which has been shown to be a PDE4 inhibitor.[19]*P. hydropiper* also contains derivatives of apigenin and luteolin, which we have shown previously to enhance relaxation responses to SNP and forskolin in the porcine coronary artery, although these compounds also inhibit calcium-induced contractions.[14]Phosphodiesterases (PDEs) are an enzyme superfamily that have been demonstrated to catalyze the hydrolysis of intracellular second messenger molecules, including cAMP and cGMP; therefore, the inactivation of PDE will indirectly increase the level of cGMP and cAMP in cells.[20] Theobromine produced a concentration-dependent relaxation of the porcine coronary artery, which was inhibited by potassium channel inhibitors, similar to that seen with the butanol soluble fraction of *P. hydropiper*. Furthermore, both rolipram, a PDE4 selective inhibitor, which increases cAMP, and sildenafil, and PDE5 selective inhibitor, which increases cGMP, produced concentration-dependent relaxations of the porcine coronary artery, which were inhibited by potassium channel inhibitors. These data indicate that phosphodiesterase inhibitors produce relaxations of the porcine coronary artery involving activation of potassium channels. Therefore, the effect of the butanol soluble fraction of *P. hydropiper* on potassium channels may be related to the presence of phosphodiesterase inhibitors such as theobromine, and are part of the same signaling pathway. The partial inhibition of the relaxation with the potassium channel inhibitors may be due to the multiple signaling pathways affected by the cyclic nucleotides.

Conclusion

In conclusion, we have found that *P. hydropiper* extracts produce an endothelium independent relaxation of porcine coronary artery with the butanol soluble fraction being the most active. The data indicate that the relaxation pathway may be due to the presence of theobromine, or other phosphodiesterase inhibitors, leading to activation of plasma membrane potassium channels.

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Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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