
Contents lists available at ScienceDirect
European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular Pharmacology

Hypotensive activity of an n-butanol extract and their purified compounds from leaves of Phyllanthus acidus (L.) Skeels in rats

Yuttapong Leeya a, Michael J. Mulvany b, Emerson F. Queiroz c, Andrew Marston c, Kurt Hostettmann c, Chaweewan Jansakul a,⁎

a Department of Physiology, Faculty of Science, Prince of Songkla University, Hat-Yai, 90112, Thailand
b Department of Pharmacology, Aarhus University, 8000 Aarhus C, Denmark
Laboratory of Pharmacognosy and Phytochemistry, School of Pharmaceutical Sciences, University of Geneva, 30 quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland

A R T I C L E I N F O

Article history:
Received 3 February 2010
Received in revised form 26 July 2010
Accepted 14 September 2010
Available online 21 September 2010

Keywords:
Blood pressure
Thoracic aorta
Kaempferol
Phyllanthus acidus
Euphorbiaceae

A B S T R A C T

We aimed to investigate the effects, identify the active substances and establish the mechanisms involved in the hypotensive activity of an n-butanol extract from leaves of Phyllanthus acidus (PA extract). PA extract caused a decrease in blood pressure of anesthetized rats that was not modified by atropine or propranolol. PA extract caused a persistent dilatation of thoracic aortic rings preconstricted with either phenylephrine or KCl, and these effects were not modified by LNA or removal of the vascular endothelium. For phenylephrine-preconstricted aortic rings, the dilatory activity of the PA extract was not modified by atropine, propranolol or indomethacin. TEA, glybenclamide or ODQ significantly inhibited the dilatory activity of the PA extract on endothelium-denuded aortic rings. Nifedipine or a Ca2+−free medium depressed the aortic rings constrictor response to phenylephrine, and that was further augmented by the PA extract. Adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid, and kaempferol were isolated from the PA extract. Each caused a decrease in blood pressure of anesthetized rats that was not modified by atropine, propranolol or indomethacin. TEA, glybenclamide or ODQ attenuated the vasodilatory activity of adenosine whereas glybenclamide and ODQ attenuated the effect of hypogallic acid. These results suggest that the hypotensive activities of the PA extract is likely the result of the direct action of these five compounds on the blood vessels by stimulating release of nitric oxide from the vascular endothelium, in part through stimulation of soluble guanylate cyclase, and opening of KATP and Kca channels in the vascular smooth muscle.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Phyllanthus acidus (L.) Skeels is a small tropical tree belonging to the family Euphorbiaceae. Its common name is Otaheiti Gooseberry, Star Gooseberry or Mayom in Thai. It is widely cultivated throughout the country (Van Welzen and Chayamarit, 2007). In Thai Folkloric Medicine, the traditional use of the leaves of the plant, Mokkhammit et al. (1971) reported that an ethanolic extract from the stem bark of the plant showed cytotoxic activity on BC and KB cell lines. Lee et al. (2006) reported that a methanolic extract from P. acidus was as a decoction given orally for treatment of hypertension (www.tungsong.com 2005). However, scientific investigations to confirm these therapeutic claims are rare. Dekker (1908) was the first scientist to investigate the chemical constituents of the stem bark of the plant and identified lupeol. Several decades later stem bark was found to contain P. acidus sterols (Ultee, 1933), beta-aminor and phyllanthol (Sengupta and Mukhopad-hyay, 1966). The root of P. acidus contains phyllanthusol A and B (Durham et al., 2002; Vongvanich et al., 2000). For pharmacological studies of the plant, Mokkhammit et al. (1971) reported that an ethanolic extract from the stems did not show any toxicity to rats, and a crude extract from the leaf and the branch showed little antibacterial activity. Lee et al. (2006) reported that a methanolic extract from P. acidus had a hepatoprotective effect on rats with acute liver damage induced by carbon tetrachloride and Vongvanich et al. (2000) found that phyllanthusols A and B, isolated from the root of the plant showed cytotoxic activity on BC and KB cell lines. Recently, P. acidus has been investigated as a potential treatment for cystic fibrosis (Sousa et al., 2007). In Thai herbal medicine, the traditional use of the leaves of the P. acidus is as a decoction given orally for treatment of hypertension (www.tungsong.com 2005). However, there is still no sound scientific evidence to support this therapeutic claim. Thus, the present study aimed at investigating whether an n-butanol extract (organic part) of a decoction from leaves of P. acidus had a hypotensive activity in anesthetized rats and subsequently to try and identify the substance(s) that were responsible for any hypotensive activity and assess the mechanism(s) involved.
2. Material and methods

2.1. Plant material

Fresh leaves of Phyllanthus acidus (P. acidus) were collected in Songkla province, Thailand. Authentication was achieved by comparison with the herbarium specimen in the Department of Biology Herbarium, Faculty of Science, Prince of Songkla University, Thailand, where a voucher specimen (Collecting No. 2548-01) of the plant material has been deposited.

2.2. Extraction and isolation

Fresh leaves of P. acidus (100 kg) were simmered in hot filtered water for a period of 3 h. The clear solution was collected and heated at 50 °C to reduce the volume to 50%. The concentrated solution was partition extracted with water-saturated n-butanol. The n-butanol phase was collected and evaporated to dryness in vacuo and lyophilized to obtain a yellow brown powder (506 g) of P. acidus extract (PA extract).

Using hypotensive guided fractionation, the PA extract (500 g) was partition extracted with CHCl₃, followed by ethyl acetate. The ethyl acetate soluble part and insoluble part were collected and evaporated. The ethyl acetate insoluble part was subjected to column chromatography over silica gel 100 (0.063–0.200 mm, 850 g) and eluted with a gradient of CHCl₃–CH₃OH from 100% CHCl₃ to 100% CH₃OH, yielding 3 fractions (A1–A3) on the basis of thin layer chromatography (TLC: gel 60 F₂₅₄ Al sheets, Merck, detection at 254 and 365 nm, CHCl₃–CH₃OH = 8:2 as a mobile phase). The hypotensive fraction, A3, was re-chromatographed on silica gel 60 (0.040–0.063 mm) and eluted with a gradient of CHCl₃–CH₃OH from 100% CHCl₃ to 100% CH₃OH, yielding 4 fractions (B1–B4). The hypotensive fraction, B3, was further fractionated by silica gel reversed phase C₁₈ column column chromatography using gradient elution of CH₃OH–H₂O: from 10% CH₃OH to 80% CH₃OH increasing each step by 10% CH₃OH and using 2.5 L of each concentration. This yielded 2 hypotensive compounds, which were identified as adenosine (355.9 mg) and caffeic acid (40.8 mg) respectively.

The ethyl acetate soluble part was subjected to column chromatography over silica gel 100 (850 g) and eluted with a gradient of CHCl₃–CH₃OH from 100% CHCl₃ to 100% CH₃OH, yielding 4 fractions (C1–C4). The hypotensive fraction, fraction C1, was further separated by MPLC on a LiChroprep® RP₁₈ column (70 × 460 mm, 40–63 µm, Merck), eluted with a gradient of CH₃OH–H₂O–H₂O: 0.05% TFA at a flow rate of 10 ml/min controlled by a UV 254 (Buchi 681 pump equipped with a Knauer UV detector), and yielded the compounds 4-hydroxybenzoic acid (100.4 mg), hypogallic acid (355.9 mg) and kaempferol (697.4 mg).

The pure compounds were characterized by mass spectroscopy on a TSQ-700 triple stage quadrupole instrument (Finnigan MAT, San Jose, CA, USA), and ¹H and ¹³C NMR spectra which were recorded on a Varian Inova 500 spectrometer (Varian, Palo Alto, CA, USA) (500 MHz and 125 MHz, respectively) in either DMSO-d₆ or CDCl₃, chemical shifts reported in ppm as δ relative to Me₄Si (internal standard).

Adenosine (1) is a white amorphous powder. For MS, ¹H and ¹³C NMR, see Aldrich, 1992.

4-hydroxybenzoic acid (2) is a white amorphous powder. For MS, ¹H and ¹³C NMR, see Li et al., 2003.

Caffeic acid (3) is a white amorphous powder. For MS, ¹H and ¹³C NMR, see Schmutz et al., 1993.

Hypogallic acid (4) is a white amorphous powder. For MS, ¹H and ¹³C NMR, see Choudhary et al., 2008.

Kaempferol (5) is a yellow amorphous powder. For MS, ¹H and ¹³C NMR, see Reddy et al., 2009.

The PA extract, as well as the five isolated active compounds were analyzed by high performance liquid chromatography (HPLC) in order to obtain a chemical profile. Analytical HPLC was carried out on a HP 1100 system equipped with a photodiode array detector (Agilent Technologies). The extract was analyzed on a Symmetry® C₁₈ column (5 µm, 3.9 × 150 mm i.d.; Waters), with a gradient of CH₃OH: H₂O + 0.05% of trifluoroacetic acid (10:90 → 100:0). The flow rate was 1 ml/min; the UV traces were measured at 210 and 254 nm and UV spectra (DAD) were recorded between 200 and 500 nm. The HPLC chromatograms together with the corresponding UV spectra of the PA extract and the five pure compounds are shown in Fig. 1.

2.3. Pharmacological studies

Adult female Wistar rats weighing 210–250 g were supplied from the Animal House, Faculty of Science, Prince of Songkla University. The animals were housed in controlled environmental conditions at 25 °C on a 10 h dark and 14 h light cycle and allowed access to standard food and tap water ad libitum. The animal methods employed in this study were approved by the Prince of Songkla University Animal Care and Use Committee. The investigation conformed to the Guide for the Care and Use of Laboratory Animals.

2.3.1. In vivo preparation

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg, i.p.). The tracheal tube was cannulated with a polyethylene tube to maintain airway patency and the animals breathed room air spontaneously. A polyethylene catheter was cannulated through the right common carotid artery which was connected to a pressure transducer (P23 ID, Gould Statham Instrument, Hato Rey, Puerto Rico) and connected to a Grass polygraph (model 7D, Grass Instrument, Quincy, MA, U.S.A.) for systemic blood pressure monitoring, and the heart rate was recorded by using a tachograph driven by the blood pressure wave. Another polyethylene tube was cannulated through the left jugular vein for intravenous administration of drugs.

The animal was then equilibrated for at least 40 min. After the equilibration period and the systemic blood pressure had reached a steady state, the dose–response relationship to the PA extract (0.3–100 mg/kg) was determined by injection of the drug through the left jugular vein using a volume not exceeding 0.1 ml for each dose and flushed with 0.1 ml normal saline, both before and after pretreating the animals with atropine (0.6 mg/kg) or propranolol (0.6 mg/kg).

With another set of animals, after equilibration of the animal for 40 min, the dose–response relationship to adenosine (0.03–0.3 mg/kg), 4-hydroxybenzoic acid (1–10 mg/kg), caffeic acid (1–10 mg/kg), hypogallic acid (1–10 mg/kg) or kaempferol (1–10 mg/kg) were determined.

2.3.2. In vitro preparation

Adult female Wistar rats weighing 210–250 g were killed by decapitation with a guillotine. The thoracic aorta was removed and carefully cleaned of adhering fat and connective tissue. Two adjacent rings of 4–5 mm in length were cut. In one ring the endothelium layer was removed mechanically by gently rubbing the intimal surface with a stainless steel rod, using the method of Jansakul et al. (1989). The aortic rings with or without a functional endothelium were mounted horizontally between two parallel stainless steel hooks taking extreme care not to damage the endothelium of the endothelium–intact aortic rings, and suspended in a 20-ml organ bath containing Krebs–Henseleit solution of the following composition (mM): NaCl 118.3, KCl 4.7, CaCl₂ 1.9, MgSO₄·7H₂O 0.45, KH₂PO₄ 1.18, NaHCO₃ 25.0, glucose 11.66, Na⁺EDTA 0.024 and ascorbic acid 0.09, maintained at 37 °C and continuously bubbled with 95% O₂ and 5% CO₂ mixture. One of the hooks was fixed at the bottom and the other was connected to a force displacement transducer that was connected to a Grass polygraph for the recording of changes in isometric tension. Prior to addition of drugs, tissues were equilibrated for 60 min under a resting tension of 1 g and the bath solution was replaced with pre-warmed oxygenated Krebs–Henseleit solution every 15 min.
Fig. 1. HPLC chromatogram of *P. acidus* extract (PA extract), and adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol, isolated from the PA extract. The column eluant from the PA extract and the isolated compounds were scanned at the wavelength 254 nm.
After equilibration, the presence of a functional endothelium of the thoracic aortic rings was assessed in all preparations as follows: the thoracic aortic ring was preconstricted with 3 μM phenylephrine until the response had reached a plateau (5–10 min), and the dilatory response to 30 μM acetylcholine was recorded. The experiment was then continued only when there was no dilatory response by the endothelium-denuded thoracic aortic rings, and at least an 80% vasodilatation to acetylcholine for the endothelium-intact thoracic aortic rings. The preparations were then washed several times with Krebs–Henseleit solution, and allowed to fully relax for 45 min before the experimental protocol began.

After the re-equilibration, the thoracic aortic rings with and without endothelium were again preconstricted with 3 μM phenylephrine or 40 mM KCl for 5–10 min. When the contraction reached a steady state, a cumulative concentration–response relationship curve was constructed to the PA extract (0.1–30 mg/ml) or vehicle (corresponding amount of DMSO). Then after several washings and a re-equilibration period of 45 min, the thoracic aortic rings were challenged with 3 μM phenylephrine or 40 mM KCl for 10 min (plateau reached) followed by several washings. This procedure was repeated every 45 min until its contractile responses to the phenylephrine or the KCl returned to the same magnitude as that of the control (before challenging with PA extract), but no more than 4 consecutive repeats.

Using another set of animals, the endothelium-intact thoracic aortic rings in the presence or absence of LNA was challenged with 3 μM phenylephrine for 10 min (plateau reached) followed by several washings, and re-equilibration for 45 min. The thoracic aortic rings were then pre-incubated with nifedipine (1 μM), or the suspending solution replaced with Ca²⁺-free Krebs solution for 30 min, and then challenged with 3 μM phenylephrine for 10 min, followed by several washings and re-equilibration for 45 min. Then the same procedures were repeated by adding the PA extract into the incubation medium to together with the nifedipine or into the Ca²⁺-free Krebs solution and incubating for 30 min before adding 3 μM phenylephrine into the incubating medium and recording the isometric tension developed over 10 min. The steady state value, at the end of drug application, was measured for experiments concerning with nifedipine, whereas the maximal transient phasic contraction was measured for the experiment performed in Ca²⁺-free Krebs solution.

Using another set of animals, the thoracic aortic rings with or without endothelium were pre-incubated with N⁵-nitro-l-arginine (LNA, 0.3 mM), atropine (10⁻⁷ M), propranolol (10⁻⁷ M), indomethacin (10⁻⁶ M), tetraethyl ammonium (TEA, 1 mM), glybenclamide...
(10^{-5} M) or 1H-[1,2,4]oxadiazolo[4,3-a]quinazoline-1-one (ODQ, 10^{-5} M) for 30 min, then each thoracic aortic ring was preconstricted with phenylephrine (3 μM) for 5–15 min (plateau reached), and the cumulative concentration–response relationship to PA extract was obtained in the presence of the corresponding blockers.

Using the same protocol as above, another set of thoracic aortic rings with or without endothelium were preconstricted with 3 μM phenylephrine for 5–15 min, followed by a cumulative concentration–response relationship to adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid, or kaempferol in the absence or presence of LNA, TEA, glybenclamide or ODQ. Another set of thoracic aortic rings with and without endothelium were preconstricted with 3 μM phenylephrine for 5–15 min, followed by a cumulative concentration–response relationship to the 5 pure compound-cocktail (adenosine 14.41%, caffeic acid 1.65%, 4-hydroxybenzoic acid 15.0%, hypogallic acid 40.69% and Kaempferol 28.25%).

2.4. Drugs

The following drugs were used: acetylcholine chloride, atropine sulphate, glybenclamide, indomethacin, nifedipine, 1H-[1, 2, 4] oxadiazolo[4,3-a]quinazoline-1-one (ODQ), phenylephrine hydrochloride,
solution, or Ca²⁺ free Kreb’s solution and PA extract on contractile responses of the Kreb’s solution, and † NaH₂P₀₄ 0.19 g/l and ascorbic acid 0.03 g/l, and the remaining stock monium (TEA), adenosine, 4-hydroxybenzoic acid, caffeic acid, hypo-
edothelium-intact (Endo) thoracic aortic rings to phenylephrine (3
propranolol hydrochloride, N⁶-nitro-arginine (LNA), tetraethylam-
monium (TEA), adenosine, 4-hydroxybenzoic acid, caffeic acid, hypo-
gallic acid and kaempferol. All drugs were purchased from Sigma, U.S.A.
LNA and TEA were dissolved in distilled water, acetylcholine chloride and atropine sulphate were dissolved in a solution containing NaCl 9 g/l, NaH₂P₀₄ 0.19 g/l and ascorbic acid 0.03 g/l, and the remaining stock solutions were initially dissolved in 10% DMSO but their further serial dilutions were made in normal saline.

2.5. Statistical analysis

Results are expressed as means ± S.E.M. of 6 animals (n = 6). Vasorelaxation was expressed as a mean ± S.E.M. of the percentage relaxation from phenylephrine (3 μM) or KCl (40 mM) preconstric-
tion levels and “n” represents the number of thoracic aortae, each one from a different animal. Statistical differences were determined by the Student’s paired or unpaired t-test or one way ANOVA. A P value < 0.05 was considered to be significant in all experiments.

3. Results

3.1. Effect of the PA extract, adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol on the mean arterial blood pressure and heart rate of anesthetized rats

An intravenous injection of the PA extract (0.3–100 mg/kg) caused a decrease in both the mean arterial blood pressure and heart rate in anesthetized female rats in a dose-dependent manner (Fig. 2). The hypotensive and negative chronotropic effects of the PA extract were not modified by pretreatment of the animals with atropine (0.6 mg/kg), a muscarinic receptor antagonist or propranolol (0.6 mg/kg), a β-adrenoceptor antagonist.

As shown in Fig. 3, adenosine (0.03–0.3 mg/kg) caused a marked decrease in the mean arterial blood pressure and heart rate in a dose-dependent manner, whereas 4-hydroxybenzoic acid (1–10 mg/kg) caused only a slight decrease in the mean arterial blood pressure and heart rate. In the case of caffeic acid (1–10 mg/kg), hypogallic acid (1–10 mg/kg) and kaempferol (1–10 mg/kg) only the highest dose, 10 mg/kg, caused a decrease in the mean arterial blood pressure and heart rate of anesthetized rats.

3.2. Investigation on thoracic aortae in vitro

3.2.1. Effects of PA extract on thoracic aortae in vitro

The PA extract (0.1–30 mg/ml) caused an endothelium-independent dilatation of thoracic aortic rings preconstricted with either phenyl-
ephrine [EC₅₀ and 95% confidence limit: 4.39 (3.88–4.98)] or KCl [(EC₅₀: 5.13 (4.61–5.71)] in a concentration-dependent manner, where-
as the vehicle (DMSO) did not show any effect (Fig. 4A, B, and E). N⁶-
nitro-arginine (LNA), a nitric oxide synthase inhibitor did not modify the vasodilatory activity of the PA extract preconstricted with either phenylephrine or KCl (Fig. 4A, B). In addition, the relaxing effect of the PA extract on the thoracic aortic rings persisted for at least 2 h after completing the PA extract cumulative concentration–response curve and the subsequent washings (Fig. 4C, D) whereas with the vehicle control groups, the aortic rings had recovered to be normally responsive to the phenylephrine-induced vasoconstriction after only 45 min.

3.2.2. Effects of calcium on thoracic aortae in vitro

Phenylephrine produced a phasic, followed by a steady tonic contraction of the endothelium-intact thoracic aortic rings no matter whether LNA was present or not. Nifedipine (Fig. 5A, tonic constraction), or Ca²⁺ free Krebs solution (Fig. 5B, phasic constraction) caused a reduction in contractile responsiveness of the thoracic aortic rings to phenylephrine. When the PA extract was also added into the incubation medium, the contractile responsiveness of the thoracic aortic rings to phenylephrine was further depressed.

3.2.3. Effects of atropine, propranolol, indomethacin, TEA, glybenclamide or ODQ on responses to PA extract on thoracic aorta in vitro

Atropine, propranolol or indomethacin did not significantly modify the concentration–response curve of the PA extract (data not shown). As shown in Fig. 6, in the endothelium-intact thoracic aortic rings, TEA showed a slight inhibition of the vasodilatory effect at low concentrations of the PA extract but this was not statistically significant. On the other hand, a slight potentiating effect was observed when the concentration of the PA extract was progressively increased, however this effect disappeared after incubating the aortic ring with LNA. In addition, when the vascular endothelium was removed, TEA caused a small inhibition of the vasodilatory activity of the thoracic aortic ring. Glybenclamide did not modify the vasodila-
tory activity of the PA extract of the thoracic aortic rings with intact endothelium. For the thoracic aortic rings without endothelium, glybenclamide caused a significant rightward shift of the PA extract concentration–response curve (Fig. 6D). ODQ caused a significant decrease in the sensitivity of the vasodilatory responses to PA extract on phenylephrine constricted thoracic aortic rings both with and without endothelium (Fig. 6E, F). In addition, there was a more pronounced rightward shift of the concentration–response curve when glybenclamide and ODQ were added together to the endothelium-denuded aortic rings (Fig. 6H).

3.2.4. Effects of adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic and kaempferol on thoracic aorta in vitro

As shown in Figs. 7–11 and Table 1, adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol caused dilatation of thoracic aortic rings preconstricted with phenylephrine in a concentration-dependent manner. Removal of the vascular endothelium, or
pre-incubating the thoracic aortic rings with LNA, caused a significant rightward shift of the concentration–response curves with an increase in the EC50 values, except that of the kaempferol on the endothelium-denuded thoracic aortic rings. The vasodilatatory activity of these compounds returned to normal within 45 min (data not shown), except that for kaempferol when the effect persisted for up to 3 h (Fig. 11C). Glybenclamide only inhibited the vasodilatatory effect of hypogallic acid on the endothelium-denuded thoracic aortic rings preconstricted with phenylephrine. In contrast ODQ caused a significant rightward shift of the concentration–response curves with an increase in EC50 values to adenosine, 4-hydroxybenzoic acid, caffeic acid and hypogallic acid on the endothelium intact thoracic aortic rings preconstricted with phenylephrine. When the endothelium of the thoracic aortic ring was removed, the inhibitory effect of the ODQ persisted with the adenosine and the hypogallic acid, but not with the 4-hydroxybenzoic acid and caffeic acid. TEA did not modify the vasodilatory effect of these 5 pure compounds on the thoracic aortic rings preconstricted with phenylephrine, except that
for adenosine where it caused a parallel rightward shift of the vasodilator curve of the endothelium-denuded ones. When the 5 pure compounds were mixed together in the same proportion to that found in the PA extract, the cocktail compounds also caused an endothelium independent dilatation of the thoracic aortic ring preconstricted with phenylephrine and its effect persisted for up to 3 h (Fig. 12A, B).

4. Discussion

4.1. Effect of the PA extract, adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol on the mean arterial blood pressure and heart rate of anesthetized rats

The present study demonstrates that PA extract exerts a hypotensive and negative chronotropic effect on rats. These effects were not modified by atropine or propranolol, indicating that the hypotensive and negative chronotropic activities of the PA extract are unlikely to be due to the components acting through the muscarinic or β-adrenoceptors of the cardiovascular system. The finding that PA extract caused a lowering of blood pressure with a decrease in heart rate, indicated that the PA extract might have effects centrally that antagonize the tachycardiac baroreceptor reflex. These were induced by its hypotensive effect, acting directly at the cardiovascular regulation region of the central nervous system and/or a peripheral effect at the vasculature and heart to decrease the blood pressure and heart rate. The present study, however, is concentrated on its effect on the blood vessels as described in the following section.

Bioguided isolation of the pharmacologically active compounds in the PA extract led to the identification of five compounds identified by classical spectroscopic methods: adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid, and kaempferol. Although these compounds are not new discoveries, this is the first report that identifies them as bioactive chemical constituents of the leaves of P. acidus. As shown in Fig. 3, all these compounds exert hypotensive and negative chronotropic activities on anesthetized rats, and adenosine has the highest potency for decreasing the blood pressure and heart rate. This result is consistent with the literature (Barraco et al., 1987; Stella et al., 1993; Suzuki et al., 2002). These findings indicate that the hypotensive and negative chronotropic activities of the PA extract is the result of synergy between these five compounds. Among them, adenosine has been extensively studied for its effect on blood pressure and heart rate by many researchers, and it is well-known that

![Graphs showing the effect of adenosine on blood pressure and heart rate](image-url)
Adenosine produced hypotension and bradycardia. These effects are thought to be mediated at adenosine receptors localized at the cardiovascular regulation regions of the hindbrain, including the nucleus tractus solitarius and in the periphery through different adenosine receptor subtypes (Barraco et al., 1988; Shryock and Belardinelli, 1997).

Fig. 8. Effect of N-nitro-L-arginine (LNA, 3 mM), endothelium, or 1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one (ODQ, 10 μM) on the dilatation of the endothelium-intact (A and C) or -denuded (B and D) thoracic aortic rings preconstricted with phenylephrine (3 μM) to 4-hydroxybenzoic acid. Each point represents the mean ± S.E.M. of 6 experiments (n = 6). * Significantly lower than those of their control group.

Fig. 9. Effect of N-nitro-L-arginine (LNA, 3 mM), endothelium, or 1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one (ODQ, 10 μM) on the dilatation of the endothelium-intact (A and C) or -denuded (B and D) thoracic aortic rings preconstricted with phenylephrine (3 μM) to caffeic acid. Each point represents the mean ± S.E.M. of 6 experiments (n = 6). * Significantly lower than those of their control group.
4.2. Investigation on thoracic aortae in vitro

4.2.1. Effects of PA extract on thoracic aortae in vitro

The PA extract caused an endothelium-independent vasodilatation of the thoracic aortic rings preconstricted with phenylephrine or with KCl. Although LNA did not modify the PA extract concentration–response curve of the endothelium-intact thoracic aortic rings, the role of NO on the vasodilatory activity of the PA extract cannot be totally excluded. The reason is that the PA extract may contain several active substances that might have different vasodilatory potencies and/or an ability to stimulate release of the NO. The finding that PA extract also caused vasodilatation in the thoracic aortic rings preconstricted with KCl indicated that the vasorelaxant effect of the PA extract is non-specific, its effect could be antagonized via an α<sub>1</sub>-adrenoceptor-mediated vasoconstriction, as well as by a voltage-mediated vasoconstriction. In the present study, we also found that the inhibitory effect of the PA extract on the contractile response of the endothelium-intact and -denuded thoracic aortic rings to phenylephrine and KCl persisted for at least 2 h, although it gradually recovered to a normal responsiveness after a periodic washing and a re-equilibration period of 45 min each (Fig. 4C and D). This effect was not found in the vehicle control group that had never been challenged with the PA extract. These results indicated that the vasodilatory effect of the PA extract is a long lasting activity. In order to avoid any misleading results due to the post-effects of the PA extract, in any experiment, a separate set of thoracic aortic rings were used: one set for the control experiment and the other for the PA extract experiments.

4.2.2. Effects of calcium on thoracic aortae in vitro

In the rat thoracic aortic rings, the α<sub>1</sub>-adrenoceptor agonist, phenylephrine induced an initial phasic contraction followed by a tonic contraction. The initial contraction is mediated by intracellular Ca<sup>2+</sup> release, whereas the sustained tonic contraction results from Ca<sup>2+</sup> influx via the voltage-calcium channels (Abebe et al., 1990; Akata, 2007; Nelson et al., 1988). In the present study, PA extract completely antagonized the phenylephrine-induced constriction of the thoracic aortic rings. Thus, it is possible that the PA extract may also play a role as a Ca<sup>2+</sup>-channel inhibitor. To examine this possibility, we used nifedipine to block the voltage Ca<sup>2+</sup>-channel.

Fig. 10. Effect of N-nitro-L-arginine (LNA, 3 mM), endothelium, glybenclamide (Glyben, 10 μM), or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10 μM) on the dilatation of the endothelium-intact (A, C and E) or -denuded (B, D and F) thoracic aortic rings preconstricted with phenylephrine (3 μM) to hypogallic acid. Each point represents the mean ± S.E.M. of 6 experiments (n=6). * Significantly lower than those of their control group.
apparatus to calcium could not be ruled out, and a further study would be needed to clarify this possibility.

4.2.3. Effects of atropine, propranolol, indomethacin, TEA, glibenclamide or ODQ on responses to PA extract on thoracic aortae in vitro

The finding that propranolol or atropine did not modify the concentration-vasodilatory effect of the PA extract confirmed that this activity of the PA extract did not involve the β-adrenoceptors or the muscarinic receptors of the blood vessels.

Some prostaglandins such as prostacyclin or prostaglandin E normally produced by the cyclo-oxygenase pathway can relax the vascular smooth muscle. Thus, it is possible that the vasodilatory activity of the PA extract may be due to components in the PA extract that stimulate release of these vasodilatory prostaglandins. However, this was not the case as it was found that indomethacin did not modify the concentration–response curves of the PA extract on the thoracic aortic rings either with or without endothelium.

TEA inhibited the vasodilatory activity of the PA extract on the endothelium-denuded thoracic aortic ring, whereas a potentiating effect was found on the endothelium-intact aortic ring and this effect was abolished by LNA. These findings indicate that the PA extract could play a role in the opening of the Ca$^{2+}$-sensitive K$^+$-channels (KCa) in the vascular smooth muscle but then this activity is overcome by nitric oxide generated from the vascular endothelium. Glibenclamide caused a significant inhibition of the PA extract vasodilatory activity only on endothelium-denuded thoracic aortic rings. This indicated that the PA extract may also have a secondary effect on the smooth muscle of the blood vessels by opening ATP-sensitive K$^+$-channels (KATP). ODQ inhibited relaxation on both the endothelium-intact and -denuded thoracic aortic rings, indicating that the PA extract may also have a secondary effect on the blood vessels by activating the soluble guanylate cyclase which then increased the cGMP level to promote its direct vasodilatory activity. The finding that when both glibenclamide and ODQ were added together there was an increased inhibitory effect of the PA extract on the vasodilatory activity but only for the endothelium-denuded thoracic aortic rings, confirmed that the PA extract could act via the smooth muscle to open the K$_{ATP}$ channel.

4.2.4. Effects of adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol on thoracic aortae in vitro

All these compounds caused a concentration-dependent dilatation of the thoracic aortic rings preconstricted with phenylephrine. Adenosine is the most potent vasodilator and is about 3 fold more potent than caffeic acid, hypogallic acid, and kaempferol, and about 12 fold more potent than 4-hydroxybenzoic acid (Table 1). The vasodilatory activity of these compounds is in the same range as previous reports (Andriambeloson et al., 1998; Cicala et al., 2003; Hourani et al., 2001; Moritoki et al., 1990; Padilla et al., 2005; Perez-vizcaino et al., 2002; Xu et al., 2007), except that this is the first report on the hypotensive and vasodilatory activity of hypogallic acid. The finding that LNA or removal of the vascular endothelium causes a rightward shift of the curve produced by these compounds indicates that these compounds act directly on the blood vessels to cause vasodilatation, as well as indirectly by stimulating the release of nitric oxide from the vascular endothelium to promote vasodilatation. This is also consistent with previous reports (Andriambeloson et al., 1998; Benkhalti et al., 2003; Moritoki et al., 1990; Taubert et al., 2002).

Glibenclamide pretreatment significantly inhibited only the hypogallic acid-induced vasorelaxation, but not by the other pure compounds. In addition, the inhibitory effect of glibenclamide occurred only on the endothelium-denuded thoracic aortic rings. These together indicate that hypogallic acid can also play a role in opening the K$_{ATP}$ channels of the vascular smooth muscle of the thoracic aortic rings. ODQ caused a parallel right shift of the
Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>EC50 (μM): 95% confidential limit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control + LNA + Glyben + ODQ + TEA</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0.15 ± 0.019 (0.13–0.18)</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>1.78 ± 0.32 (1.49–2.13)</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.32 ± 0.023 (0.32–0.49)</td>
</tr>
<tr>
<td>Hypogallic acid</td>
<td>0.62 ± 0.072 (0.54–0.72)</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.58 ± 0.071 (0.49–0.7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compounds</th>
<th>EC50 (μM): 95% confidential limit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control + LNA + Glyben + ODQ + TEA</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0.53 ± 0.06 (0.45–0.61)</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>2.81 ± 0.32 (2.15–3.68)</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>1.96 ± 0.04 (1.65–3.21)</td>
</tr>
<tr>
<td>Hypogallic acid</td>
<td>0.65 ± 0.07 (0.63–0.7)</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.7 ± 0.08 (0.56–0.87)</td>
</tr>
</tbody>
</table>

Values were obtained from 6 experiments (n=6) for each group.

*Significantly higher than their corresponding control group.

concentration–response curves for all of these 4 pure compounds. This implies that the soluble guanylate cyclase pathway would be involved in the vasodilatation produced by these compounds. However, the soluble guanylate cyclase might be generated from nitric oxide perhaps released by these agonists, or be stimulated directly by the agonists. To unravel these possibilities, further experiments were carried out on the endothelium-denuded thoracic aortic rings to remove the endothelium nitric oxide generation. In these preparations, ODQ did not cause a shift in the vasodilator concentration–response curves of 4-hydroxybenzoic acid and caffeic acid, whereas for adenosine and hypogallic acid, the shift still persisted although it was about two fold less than those obtained from the endothelium-intact thoracic aortic rings. These results indicate that only adenosine and hypogallic acid, but not 4-hydroxybenzoic acid or caffeic acid could stimulate the soluble guanylate cyclase of the vascular smooth muscle to promote their direct effect on vasodilatation. The finding that TEA caused a rightward parallel shift of the relaxation curve for adenosine, but not for the other 4 pure compounds, and only on the endothelium-denuded thoracic aortic rings, indicated that adenosine would also cause an opening of the KCa channel of the vascular smooth muscle.

Kaempferol is a common flavonoid found in many plants. However, this is the first report of substantial amounts of kaempferol in the leaves of *P. acidus*. Kaempferol exerted an endothelium-independent vasodilatation of the thoracic aortic rings, as well as stimulating the release of the NO from the vascular endothelium. These results are analogous to those reported by Taubert et al. (2002) and also Perez-Vizcaino et al. (2002) who each suggested that the vasodilatory effect of the kaempferol was not associated with the soluble guanylate cyclase since ODQ had no effect on the concentration–response curve of quercetin, by the un-metabolised form of these preparations, ODQ did not cause a shift in the vasodilator concentration–response curves of 4-hydroxybenzoic acid and caffeic acid, whereas for adenosine and hypogallic acid, the shift still persisted although it was about two fold less than those obtained from the endothelium-intact thoracic aortic rings. This study has shown that the vasodilatory effects of the PA extract on the thoracic aortic rings are endothelium-independent, and are not modified by LNA or by the removal of the vascular endothelium. This is different from the effects produced by the five individual isolated compounds. This could be explained if these compounds separately possess differences in (1) their vasodilatory potency both directly and indirectly via stimulation of the NO release, opening of the KATP channels, opening of the KCa channels and/or stimulation of the soluble guanylate cyclase, and (2) the relative amounts of the individual compounds present in the PA extract. These amount when mixed together in the PA extract or as a cocktail of the five ingredients result in there being no difference in the vasodilatation between the endothelium-intact and endothelium-denuded thoracic aortic rings and also the effect lasted for up to 3 h. The finding that only kaempferol produced a long-lasting inhibitory effect on the phenylephrine-induced thoracic aortic ring constriction, indicates that the persistent vasodilatory activity of the PA extract is likely due to kaempferol. Other differences observed between the cocktail of the 5 isolated compounds and the PA extract can be possibly attributed to other unidentified compounds present in the extract.

![Fig. 12](image_url)

Fig. 12. (A) Dilatory responsiveness of a cocktail of the five ingredients (adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol, in the same proportion to that found in the PA extract) on endothelium-intact (Endo) and denuded (No endo) thoracic aortic rings preconstricted with phenylephrine (3 μM). And (B) maximal contractile responses of the thoracic aortic rings to phenylephrine before performing the concentration-response (C–R) curve, at the end of the C–R curve prior washing (0 min) and after a 45 min interval washing period after performing a concentration-dilatory response relationship to the cocktail and challenged with 3 μM phenylephrine. Each point represents the mean ± S.E.M. of 6 experiments (n = 6). * Significantly lower than those of the endothelium-intact thoracic aortic rings, and † significantly lower than those produced by phenylephrine before performing the C–R curve of their corresponding groups.
5. Conclusion

The present study has demonstrated that a PA extract has a hypotensive and a negative chronotropic activity in rats. These effects were not mediated via the β-adrenoceptors or the muscarinic receptors of the vascular system. The hypotensive effect of the PA extract is probably mediated directly at the blood vessel to cause vasodilatation. The bioactive principles identified as being responsible for these activities are most likely to be adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid, and kaempferol. These bioactive principles act directly on the vascular smooth muscle to cause vasodilatation, and indirectly by stimulating the release of nitric oxide from the vascular endothelium, as well as behaving as a soluble guanylate cyclase stimulator, as an ATP-sensitive K+ channel opener and/or a Ca²⁺-sensitive K⁺ channel opener to promote the vasodilatory activities of the compounds. These findings provide scientific support for the traditional uses of decoctions from P. acidus leaves in the treatment of hypertension in man.

Acknowledgements

This work was supported by the Graduate School, Prince of Songkla University, Thai Government, and Thailand Research Fund under the RQJ Ph.D Program. The authors thank Dr. Brian Hodgson for assistance with the preparation of the manuscript.

References


Dekker, S., 1908. Phytochemical notes. Pharm. Week. 45, 1156 (Abstract W-00584 from NAPRALERT; College of pharmacy, University of Illinois-Chicago, IL.).


W-00584 from NAPRALERT, College of pharmacy, University of Illinois-Chicago, IL).