CHAPTER 2

CARDIOVASCULAR EFFECTS OF AN N-BUTANOL EXTRACT FROM FRESH FRUIT OF *RANDIA SIAMENSIS*

2.1 Abstract

Randia siamensis is used in Thai Traditional medicine for controlling blood pressure and inducing abortion. The present study investigated the cardiovascular effects of an *R. siamensis* fruit extract, and mechanisms involved in anesthetized normal and reserpinized rats. *R. siamensis* extract (0.4-12 mg/kg, i.v.) increased the mean arterial blood pressure (MAP) and heart rate. Both effects were significantly inhibited by phentolamine (2 mg/kg, i.v.) or propranolol (0.6 mg/kg, i.v.). The combination of phentolamine and propranolol, or reserpine pretreatment, inhibited the positive chronotropic effect and caused a slight decrease in the MAP. *In vitro*, *R. siamensis* extract (0.001-0.3 mg/ml) increased the rate of beating of the right atrium and the strength of the electrical field-stimulated contraction of the left atrium, both effects inhibited by propranolol, or with reserpine-pretreated rats. *R. siamensis* extract (0.01-3 mg/ml) produced a contraction of isolated thoracic aorta, which was potentiated by N^G-nitro-L-arginine (LNA), or by removal of the vascular endothelium, but inhibited by phentolamine, or reserpine. The *R. siamensis* extract (0.3 -3 mg/ml) caused a relaxation of phenylephrine-preconstricted aortic rings, which was potentiated by reserpine pretreatment, and abolished after removal of the vascular endothelium, or in the presence of LNA. These results suggest that *R. siamensis* extract exerts both hypertensive and positive chronotropic effect via the α- and β-adrenergic receptors of blood vessels and the heart, due to release of catecholamines from sympathetic nerve terminals and the adrenal medulla. The hypotensive activity results from the release of nitric oxide causing dilatation of the blood vessels.

2.2 Introduction

Randia siamensis (*R. siamensis*) is a climbing tree of the subfamily Gardennieae, family Rubiaceae (Figure 15). It is widely distributed in tropical and subtropical regions, especially in Asia and Africa (Rendle, 1953). Craib (1939) reported that *R. siamensis* Craib is synonymous with *R. longiflora* Hook f., R. uncata Ridl.,

especially in Asia and Africa (Rendle, 1953). Craib (1939) reported that *R. siamensis* Craib is synonymous with *R. longiflora* Hook f., R. uncata Ridl., *Griffithia siamensis* Miq. and *Webera siamensis* Kurz. It can be found in all parts of Thailand and Burma, especially in humid regions. This plant is known by various local names in Thailand: Khat khao, Khet khao, Khat khao naam and Khat khao thuea. All parts of *R. siamensis* have been used in Thai Traditional medicine. The fruits have been used for inducing abortion and as an emmenagogue or hematinic, the leaves are claimed to control blood pressure, the root is used for antipyritic and antiscurvy activities, the flowers have been used to stop nosebleeds, and the stem part is used as a hematinic (Pongboonrod, 1959). However, scientific investigations to test these therapeutic claims are very scarce. Aukkanibutra (1985) identified the chemical constituents of fruits of this plant. She found that dried fruit of *R. siamensis* contains: ursolic acid, pseudoginsenoside-RT_1, pseudoginsenoside-RP_1 and an unidentified saponin. Later, Reanmongkol et al. (1994) reported that pseudoginsenoside-RP_1, the minor component of the plant fruit, has an antinociceptive activity similar to that of aspirin, a peripherally acting analgesic drug. However, the activity of this purified compound was much less potent than that of the crude extract. Jansakul et al. (1999) tested the activities of the pseudoginsenoside-RT_1, isolated by Aukkanibutra (1985), on the cardiovascular system and found that the pseudogensenoside-RT_1 (4-32 mg/kg) caused a decrease in mean arterial blood pressure, with an increased heart rate in a dose-dependent manner in anesthetized rats. However, the mechanisms for these effects have not yet been elucidated due to the limited availability of the substance. This led us to return to study the cardiovascular effects of the crude extract from fresh fruits of *R. siamensis* (*R. siamensis* extract). We found that the crude extract (4-12 mg/kg) caused an increase in both MAP and heart rate in a dose-dependent manner. The effect was more potent than that of the saponin pseudoginsenoside-RT_1. It is possible that the fresh fruit of *R. siamensis* contains some other substances, besides pseudoginsenoside-RT_1, that have effects on the cardiovascular system.

A

B

Figure 15 Fresh fruits (A) and the climbing tree (B) of

2.3 Objectives

To investigate the cardiovascular profile of the *R. siamensis* extract in the rat, as well as to assess the mechanisms involved, and compare the results with those produced by isoproterenol, epinephrine and norepinephrine.

2.4 Material and Methods

2.4.1 Plant material

Fresh fruits of *Randia siamensis* were collected in Songkhla Province, Thailand. Authentication was achieved by comparison with herbarium specimens in the Department of biology Herbarium, Faculty of Science, Prince of Songkla University, Songkhla Province, Thailand, where a voucher specimen of plant material has been deposited.

2.4.2 Preparation of *Randia siamensis* extract

Preparation of *R. siamensis* extract is shown in Figure 16. Fresh and unripe fruits of *R. siamensis* (5 kg) were blended and macerated three times with 80% methanol (10 liters) during a week period. The methanol extract was filtered and evaporated under reduced pressure, yielding 200.5 g of the MeOH extract. The methanol extract was dissolved in water, and a liquid/liquid partition was undertaken with chloroform, followed by n-butanol. The n-butanol phase was evaporated under reduced pressure, and the residue was lyophilized to obtain a crude yellowish powder, the *R. siamensis* n-butanol extract (*R. siamensis* extract, 126.2 g).

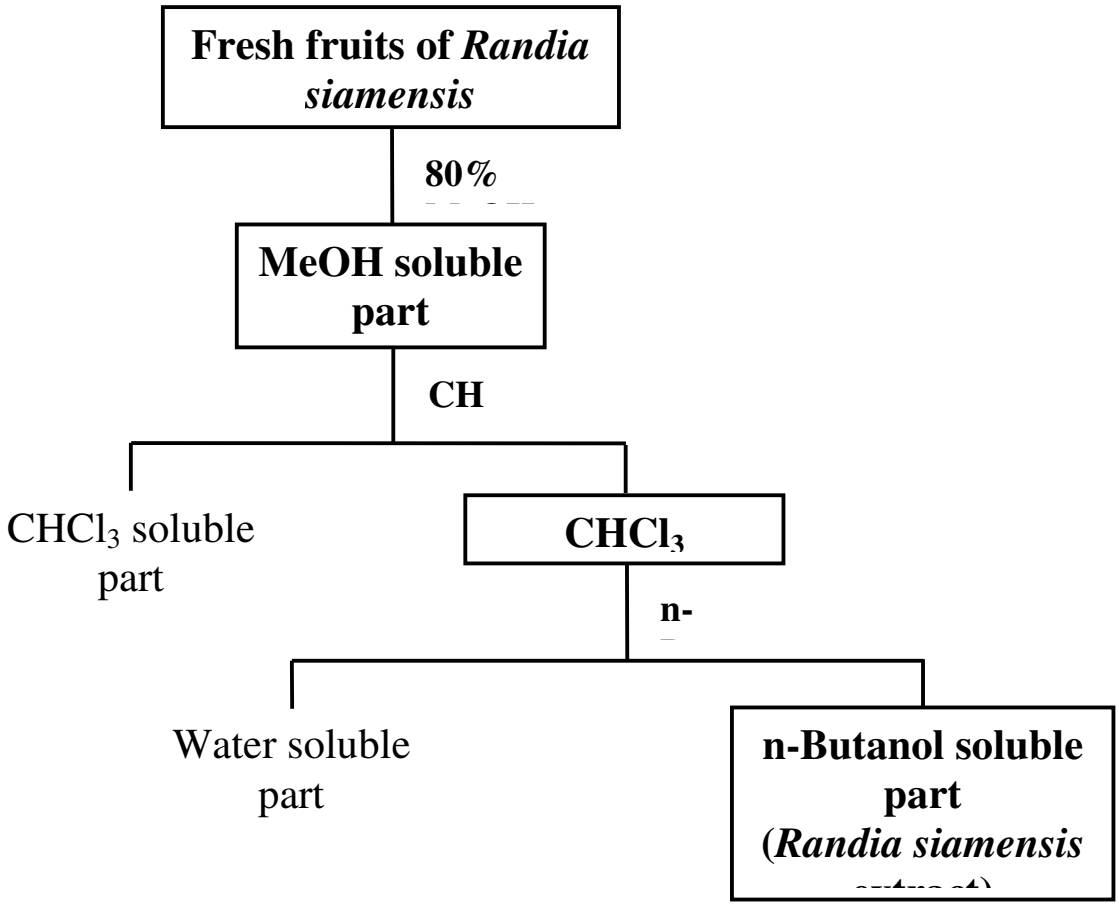


Figure 16 Flow chart showing preparation of n-butanol extract from fresh fruits of *Randia siamensis*

2.4.3 Pharmacological studies of the *R. siamensis* extract

In vivo studies

Adult female Wistar rats in estrus (220-270 g) were supplied from the Animal House, Faculty of Science, Prince of Songkla University. They were maintained in controlled environmental conditions (24-26^{o}C) with a 12 h light/dark cycle and allowed access to standard food and tap water ad libitum. Preparation of animals followed the Prince of Songkla University guidelines for the approved Care and Use of Experimental Animals.

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The tracheal tube was cannulated with a polyethylene tube to facilitate spontaneous respiration. The systemic blood pressure was recorded from the right common carotid artery via an arterial cannula connected to a pressure transducer (P23 ID, Gould Statham Instrument, Hato Rey, Puerto Rico), and the heart rate was recorded using a tachograph driven by the blood pressure wave, which were connected to a Grass polygraph (Model 7D, Grass Instrument, Quincy, MA). The animal was then equilibrated for at least 40 min before the experiment was started (Figure 17).

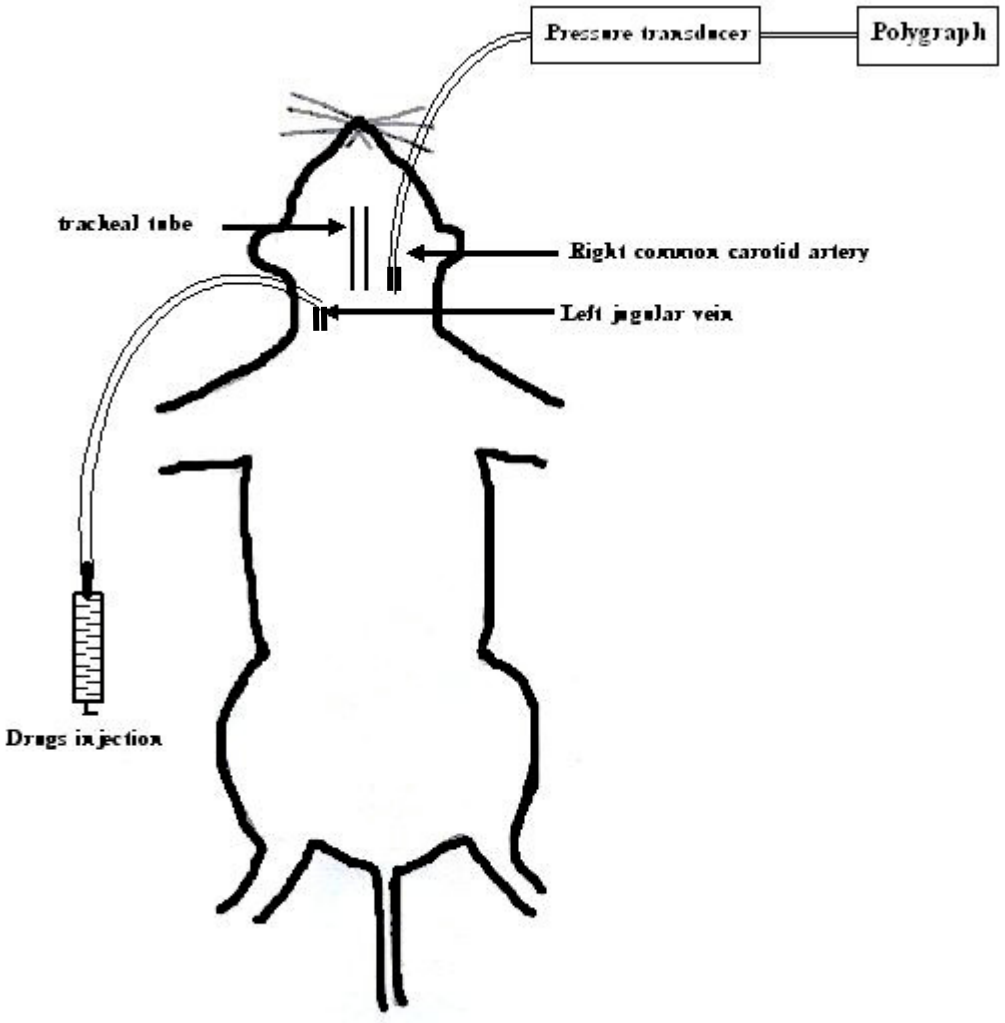


Figure 17 *In vivo* preparation of anesthetized rat

Effect of *R. siamensis* extract *on blood pressure and heart rate*

After the period of equilibration, the dose-response relationships to norepinephrine, isoproterenol, epinephrine, or *R. siamensis* extract were determined by intravenous injection into left jugular vein of a volume not exceeding 0.1 ml for each dose and flushed in with 0.1 ml saline. Each rat was used for only one agonist.

Effects of propranolol and/or phentolamine on mean arterial blood pressure and heart rate to norepinephrine, isoproterenol, epinephrine or *R. siamensis* extract

Using other sets of animals, after equilibration of the animals for 40 min, propranolol (0.6 mg/kg, i.v.), phentolamine (2 mg/kg, i.v.), or both propranolol and phentolamine were injected through the left jugular vein. After 20 min re-equilibration, the dose-response relationships to norepinephrine, isoproterenol, epinephrine, or *R. siamensis* extract were determined.

Effects of reserpine on the positive chronotropic effect and the increase in mean arterial blood pressure of the *R. siamensis* extract

Using another set of animals, the rat was treated with reserpine at a dose of 5 mg/kg, i.p., once a day starting for 2 days before the experiment (Taesotikul et al., 1998). Each experiment of reserpinized rats were firstly tested for the efficacy of reserpine treatment by an intravenous injection of 0.3 mg/kg tyramine (Hubbard et al., 1997), which the dose produced 80% of maximum increased heart rate and MAP, to reserpinized rats. The treatment of reserpine was considered to be effective when less than 20% response induced by 0.3 mg/kg tyramine was

determined. Thereafter the dose-response relationship to *R. siamensis* extract was determined using the same protocol as above.

In vitro studies

Preparation of the right and the left atrium

A normal or reserpinized rat was killed by decapitation with a guillotine. The thorax was opened, the heart was rapidly removed and placed in Krebs Heinseleit solution saturated with carbogen (95 % O_2 + 5 % CO_2) at 37^{o}C, and allowed to beat for a few seconds to expel intra-atrial chamber blood. Both the left and the right atria were excised from the ventricles, and were then separated. The right atrium was mounted in a 20-ml organ bath, one end was fixed at the bottom and the other end connected to a force-displacement transducer (FT03C) connected to a Grass polygraph, under a basal tension of 0.8 g. The rate of spontaneous atrial contraction was recorded using a tachograph. The atrium was allowed to equilibrate for 50 min with changes in Krebs solution every 15 min.

The left atrium was mounted between two platinum electrodes approximately 10 mm apart (left and right) under a basal tension of 0.5 g. The atrial tension was recorded by means of a Grass force-displacement transducer connected to a Grass polygraph. The tissues were equilibrated for 50 min. After the equilibration, the left atrium was stimulated with several trains at 3 Hz, 5 msec pulse duration with a 1 V increase in voltage for each step until the threshold voltage (3-5 V) was reached. Preparations were allowed to equilibrate for another 15 min (Figure 18).

Preparation of thoracic aorta

Immediately after an excision of the heart, the thoracic aorta was removed and dissected free of connective tissue and fat. Two adjacent rings of 4-5 mm in length were cut. In one ring, endothelium 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 were suspended horizontally between two stainless steel hooks in a 20-ml organ bath containing Krebs solution. One of the hooks was fixed to the bottom and the other was connected to a force displacement transducer 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. The Krebs solution was replaced every 15 min (Figure 18).

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 $3x10^{-6}$ M phenylephrine until the response had plateau (5-8 min), and the dilatory response to $3x10^{-6}$ M acetylcholine was recorded. The experiment was continued only when there was no dilatory response for the endothelium-denuded thoracic aortic rings, and at least 80% vasodilatation to acetylcholine for the endothelium-intact thoracic aortic rings.

The organ bath contained Krebs Henseleit solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ 7 H_2O 0.45, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.66, Na_2EDTA 0.024 and ascorbic acid 0.09, maintained at 37°C, and continuously bubbled with 95% O_2 and CO_2.

Figure 18 *In vitro* preparation of the atrium (left) and the thoracic aortic ring (right)

Effects of propranolol and/or atropine on the positive chronotropic and positive inotropic effects on the right and left atria to the *R. siamensis* extract

For the right atrium, after a 50-min equilibration, the concentration-response relationships to *R. siamensis* extract (0.001-0.3 mg/ml) on rate of spontaneous contraction of the right atria was determined before and after pre-incubation (at least 40 min for each concentration of the antagonist) with (10^{-8} and 10^{-7} M) propranolol and/or atropine.

For the left atria, after equilibration, the contraction was driven with a 10-20% suprathreshold voltage (5-7 V), allowing 5 min of continuous contraction. Then, cumulative challenge with *R. siamensis* extract (0.01-0.3 mg/ml) before and after pre-incubation (at least 40 min for each concentration of the antagonist) with propranolol (10^{-8} and 10^{-7} M) was performed. Each concentration was left for 2-3 min by which time the response reached a plateau. Inotropic responses of the drugs were calculated just before starting the construction of the concentration-response curves to the test agents.

Effects of reserpine pretreatment on the positive chronotropic and positive inotropic effects *of R. siamensis* extract on isolated atria

The efficacy of reserpine treatment was firstly tested *in vivo*. When, less than 20% response induced by 0.3 mg/kg tyramine was determined, the *in vitro* experiments on the right and left atria were started. The concentration-relationships to *R. siamensis* extract (0.001-0.3 mg/ml) on the positive chronotropic and inotropic effects of the right and left atria were determined in reserpinized rats using the same protocol as above.

Effects of the *R. siamensis* extract and phenylephrine on thoracic aortae *in vitro*

After equilibration, the thoracic aorta was challenged with $3x10^{-6}$ M norepinephrine for 10 min, by which time the maximal response had plateaued. Then followed several washings, and re-equilibration for 30 min, a cumulative concentration-response relationship of the thoracic aorta to the *R. siamensis* extract was obtained. Effects of nitric oxide or phentolamine on the thoracic constrictor response of *R. siamensis* extract were studied by using another two sets of the thoracic aorta: one without endothelium, and the other one with intact endothelium, thoracic aortic rings that had been pre-incubated with LNA ($3x10^{-4}$ M) for 40 min. Then the concentration-response relationship of the thoracic aortic rings to an *R. siamensis* extract was obtained before and after being pre-incubated (40 min for each concentration) with phentolamine (10^{-8} and 10^{-7} M). Using the same protocol, a cumulative concentration-response relationship to phenylephrine of the thoracic aortic rings was obtained in the absence or presence of LNA.

Effects of endothelium or reserpine pretreatment on vasodilator activities of *R. siamensis* extract

Dilator responses of the thoracic aorta to the *R. siamensis* extract were also studied by using endothelium-intact, endothelium-denuded, and reserpinized thoracic aortic rings. After 40 min equilibration, the aortic rings were preconstricted with $3x10^{-6}$ M phenylephrine for 5-8 min (plateau reached), and the concentration-response relationship to *R. siamensis* extract was determined.

2.4.4 Drugs and chemicals

The following drugs were used: acetylcholine chloride, atropine sulphate, (-)-epinephrine bitartrate, (□)-isoproterenol hydrochloride, phentolamine hydrochloride, phenylephrine hydrochloride, (-)- norepinephrine bitartrate, propranolol hydrochloride, reserpine,

and N^G-nitro-L-arginine (LNA). All drugs were purchased from Sigma, USA. *R. siamensis* extract and LNA were dissolved in distilled water, the remainder was dissolved in a solution (g/L): NaCl 9.0, NaH_2PO_4 0.19 and ascorbic acid 0.03.

The organic solvents for plant extraction: methanol, chloroform and n-butanol were purchased from Merck, Germany.

2.4.5 Data analysis

Data are expressed as mean ± S.E.M. of 6 experiments (n=6). Increases in force of contraction (vasoconstriction) were expressed as mean ± S.E.M. of percentage contraction of vessels compared to the maximal response obtained from $3x10^{-6}$ M norepinephrine. Decreases in force of contraction (vasodilatation) were expressed as mean ± S.E.M. of percentage relaxation of vessels from 10^{-6} M phenylephrine preconstruction-levels. Test of significance were done using the Student's paired or unpaired t-test or one-way ANOVA. In all cases, a *p* value of 0.05 or less was considered statistically significant.

2.5 Results

2.5.1 Effects of propranolol and/or phentolamine on mean arterial blood pressure and heart rate of anesthetized rats to norepinephrine, isoproterenol or epinephrine

Basal mean arterial blood pressure (MAP) and heart rate of anesthetized rats are 136 ± 9.37 mmHg and 388.33 ± 12.49 bpm respectively.

As shown in Figure 19A and B, norepinephrine, a non-specific α-adrenergic receptor agonist, caused an increase in MAP in a dose-dependent manner and a decreased heart rate but only at the highest dose (10^{-1} mg/kg). In the presence of phentolamine (2 mg/kg), the

dose-response curves of the norepinephrine on both MAP and heart rate were significantly shifted to the right.

Isoproterenol, a non specific β-adrenergic receptor agonist, caused a dose-dependent decrease in MAP with an increase in heart rate. In the presence of propranolol (0.6 mg/kg), a non-specific β-adrenergic receptor blocking agent, both the decrease in MAP and the increase in heart rate to isoproterenol were significantly inhibited (Figure 19C and D).

Small doses (10^{-5}-10^{-4} mg/kg) of epinephrine caused a decrease, while at higher doses (10^{-3}-$3x10^{-3}$ mg/kg) an increase in MAP was found. All dosages caused an increase in heart rate in a dose-dependent manner. Both the hypotensive and the positive chronotropic effects of epinephrine were inhibited by propranolol (Figure 20A and B). However, when phentolamine was given together with propranolol, the hypertensive effects of the epinephrine on the rats with propranolol was further suppressed (Figure 20C), whereas there was no further effect on heart rate (Figure 20D).

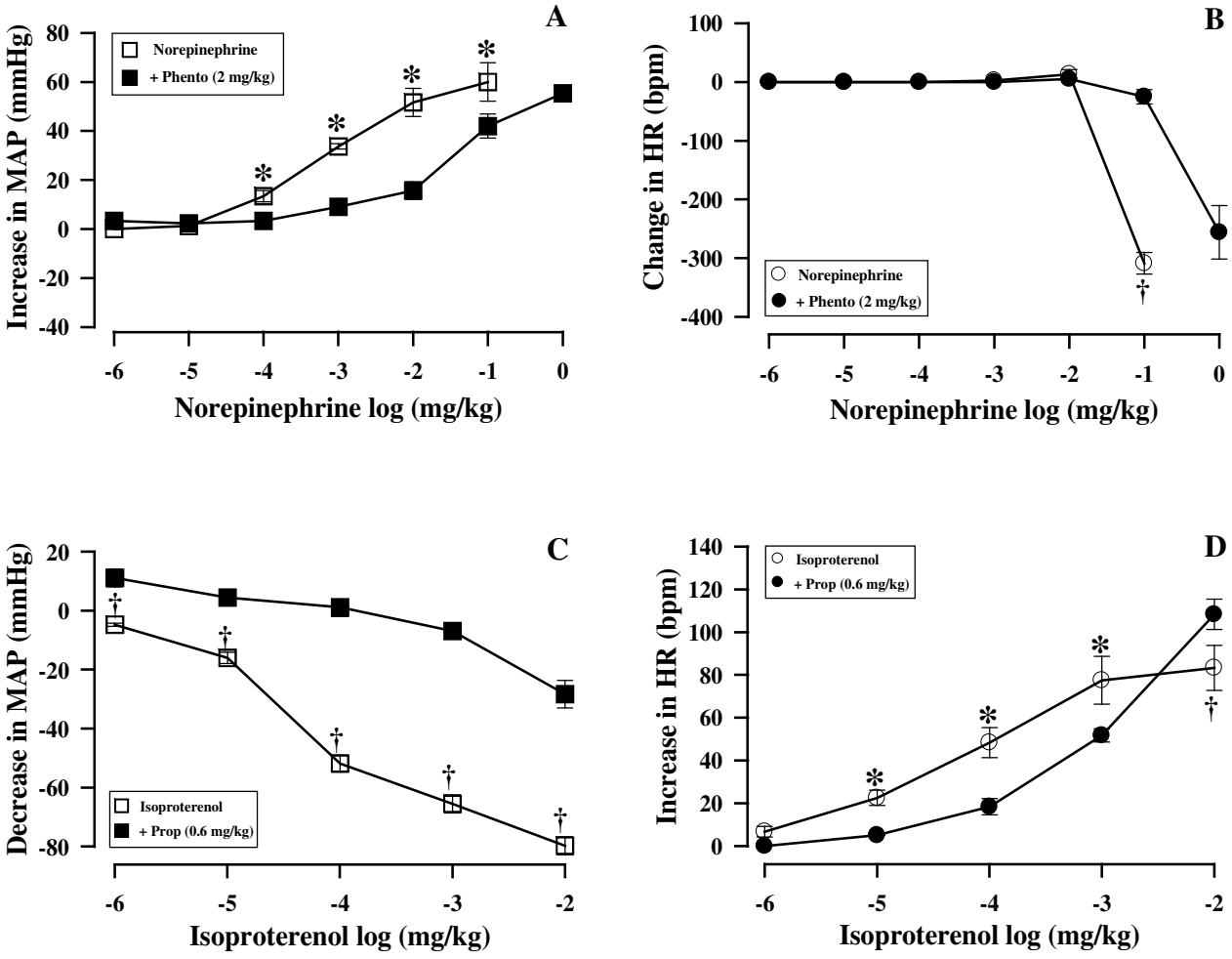


Figure 19 Effects of norepinephrine (A and B) or isoproterenol (C and D) on the mean arterial blood pressure (MAP, left) and heart rate (HR, right) before or after blocking with phentolamine (phento) or propranolol (prop) in anesthetized rats. Each point represents mean ± S.E.M. of 6 experiments. * Significantly higher than those in the presence of phentolamine or propranolol, $p<0.05$.

† Significantly lower than those in the presence of phentolamine or propranolol, $p<0.05$.

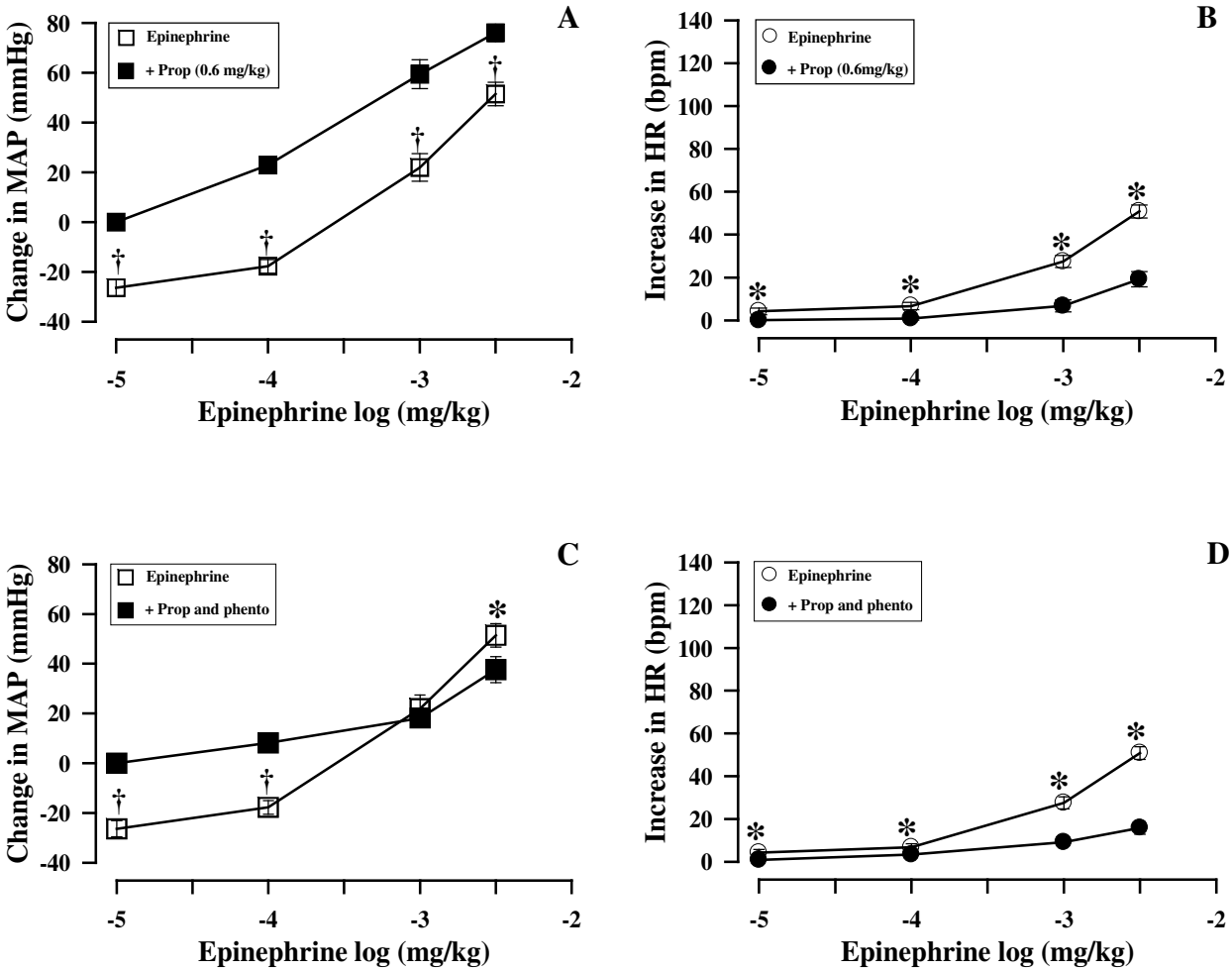


Figure 20 Effects of epinephrine on the mean arterial blood pressure (MAP, left) and heart rate (HR, right) before or after blocking with propranolol (prop), or propranolol and phentolamine (phento) in anesthetized rats. Each point represents mean □ S.E.M. of 6 experiments. ***** Significantly higher than those in the presence of propranolol, or propranolol and phentolamine, $p \square 0.05$. □ † Significantly lower than those in the presence of propranolol, or propranolol and phentolamine, $p \square 0.05$.

2.5.2 Effects of propranolol and/or phentolamine on mean arterial blood pressure and heart rate of anesthetized rats to the *R. siamensis* extract

Basal mean arterial blood pressure and heart rate of anesthetized rats are 101 □ □8.01 mmHg and 410 □ □12.91 bpm respectively.

Typical recordings of blood pressure and heart rate to *R. siamensis* extract in anesthetized rats are shown in Figure 22.

Intravenous injections of the *R. siamensis* extract (0.4-12 mg/kg) to anesthetized rats caused a dose-dependent increase in heart rate and MAP (Figure 21).
Propranolol (0.6 mg/kg) caused a rightward parallel shift of the dose-response curve of *R. siamensis* extract on the heart rate, but produced no change on the dose-response curve of the MAP. In the presence of phentolamine (2 mg/kg), however increasing amounts of extract caused a decrease in the MAP, rather than an increase, and reduced the extract-induced increase on the heart rate (Figure 21C and D). When propranolol and phentolamine were given to the animals at the same time, the dose-response curve to *R. siamensis* extract on heart rate was shifted to the right in a similar way to that caused by propranolol alone (Figure 21F), while the mean arterial blood pressure was decreased in a similar manner to that obtained with phentolamine alone (Figure 21E).

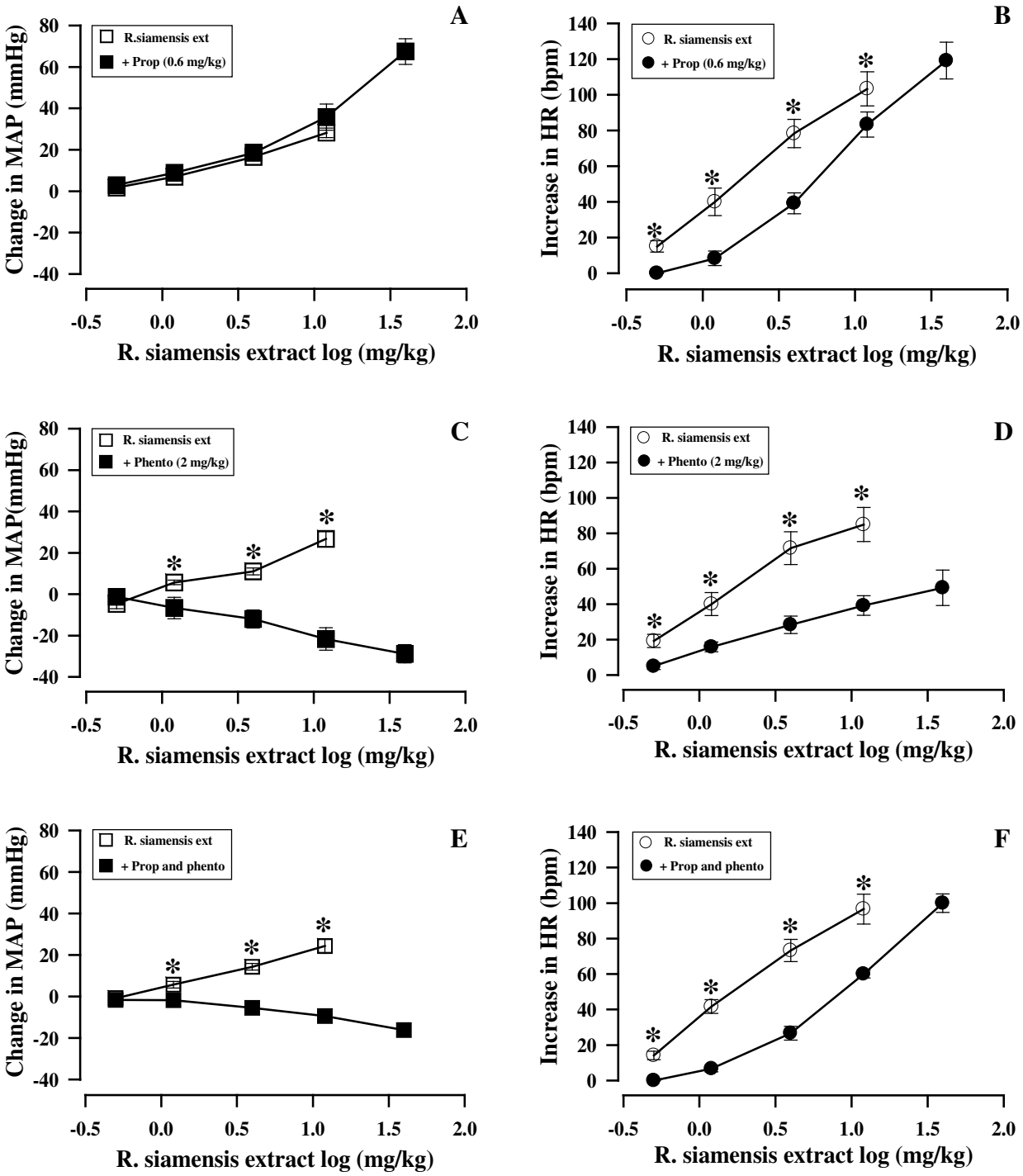

A
R.siamensis ext
+ Prop (0.6 mg/kg)
Change in MAP (mmHg)
R. siamensis extract log (mg/kg)
B
R. siamensis ext
+ Prop (0.6 mg/kg)
Increase in HR (bpm)
R. siamensis extract log (mg/kg)
C
R. siamensis ext
+ Phento (2 mg/kg)
Change in MAP(mmHg)
R. siamensis extract log (mg/kg)
D
R. siamensis ext
+ Phento (2 mg/kg)
Increase in HR (bpm)
R. siamensis extract log (mg/kg)
E
R. siamensis ext
+ Prop and phento
Change in MAP (mmHg)
R. siamensis extract log (mg/kg)
F
R. siamensis ext
+ Prop and phento
Increase in HR (bpm)
R. siamensis extract log (mg/kg)

Figure 21 Effects of propranolol (prop) and/or phentolamine (phento) on changes in the mean arterial blood pressure (MAP, left), and the positive chronotropic effect (increase in heart rate, right) of *R. siamensis* extract in anesthetized rats. Each point represents mean □ S.E.M. of 6 experiments. *****Significantly higher than those in the presence of propranolol and/or phentolamine, p □ *0.05*.

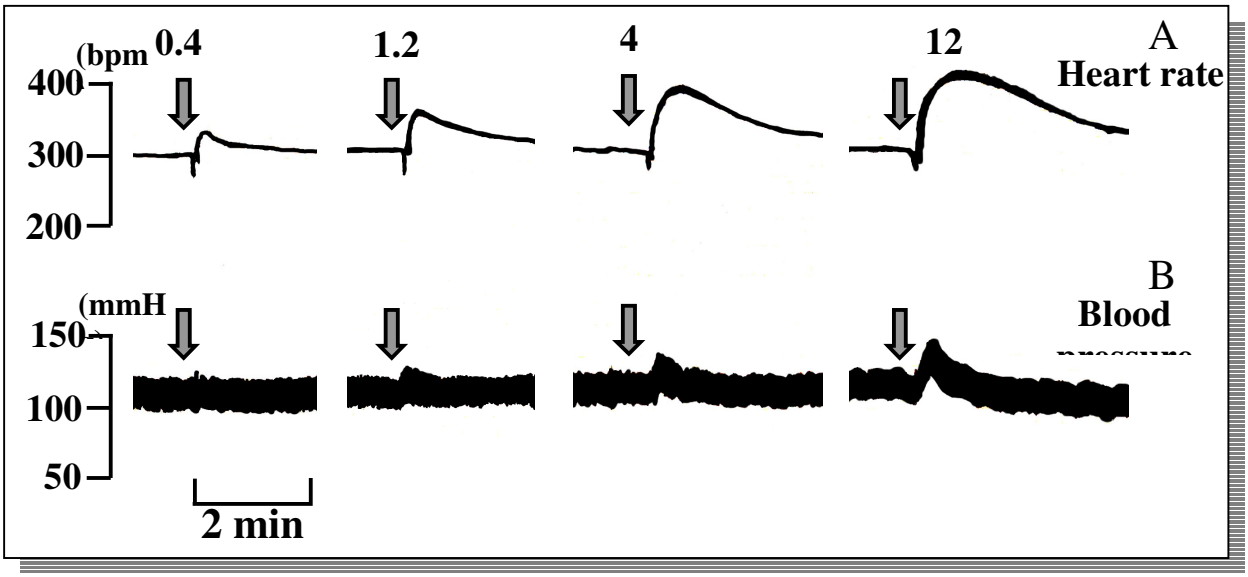


Figure 22 Typical recording showing effects of intravenous injections of the *R. siamensis* extract (0.4-12 mg/kg) on the heart rate (A) and the blood pressure (B).

2.5.3 Effects of reserpine treatment on the positive chronotropic effect and the hypertensive effect of *R. siamensis* extract

Basal mean arterial blood pressure and heart rate of anesthetized reserpine pretreated rats are 57.5 □ 4.26 mmHg and 216.67 □ □11.23 bpm respectively, which are significantly lower than those of control groups (MAP, 101.33 □ □8.01 mmHg and heart rate, 410□ □ 12.91 bpm).

Results are shown in Figure 23. *R. siamensis* extract caused an increase in heart rate and the MAP in normal rats. After treatment with reserpine the positive chronotropic effect of the *R. siamensis* extract was almost abolished. In the case of blood pressure, the *R. siamensis* extract caused a slightly decrease in MAP instead of an increase.

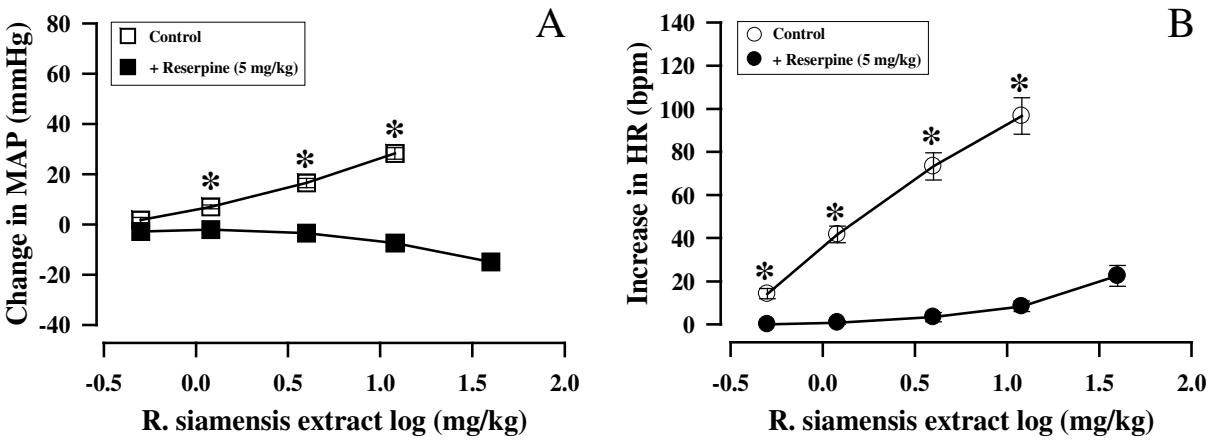


Figure 23 Effects of the depletion of sympathetic neurotransmitters by reserpine on the increase in mean arterial blood pressure (MAP, A) and the positive chronotropic effect (increase in HR, B) of *R. siamensis* extract in anesthetized rats. Each point represents mean □ S.E.M. of 6 experiments. *Significantly higher than those in reserpinized rats, p □ 0.05.

2.5.4 *In vitro* effects of propranolol and/or atropine on the positive chronotropic and positive inotropic effects of the *R. siamensis* extract

Basal rate of spontaneous contraction of the right atria are 291.67□ □ 10.14 bpm, and the basal strength of electrical field-stimulated contraction of the left atrium are 0.24 □ □0.04 g. Typical recordings of the effects of the *R. siamensis* extract on the rate of spontaneous contraction of the right atrium and the strength of electrical field-stimulated contraction of the left atrium are shown in Figure 25.

A cumulative addition of the *R. siamensis* extract (0.001-0.3 mg/ml) caused an increase in the rate of spontaneous contraction of the right atrium in a concentration-dependent manner. In the presence of 10^{-8} M propranolol, the concentration-dependent curve of the increased rate of contraction of the right atrium made a parallel shift to the right with a decrease in its maximal response. At a concentration of 10^{-7} M propranolol, the concentration -dependant response of the spontaneous contraction to the extract was almost completely suppressed. 10^{-8} M and 10^{-7} M atropine had no effect on the positive chronotropic effect of the *R. siamensis* extract (Figure 24B). In addition, when both propranolol and atropine (10^{-8} and 10^{-7} M) were applied together, the responses to the *R. siamensis* extract did not significantly change (Figure 24C).

R. siamensis extract caused an increase in the strength of the electrical field-stimulated contraction of the left atrium. Propranolol (10^{-8} M and 10^{-7} M) caused a concentration-dependent significant rightward shift of the concentration-response curves to the *R. siamensis* extract (Figure 24D).

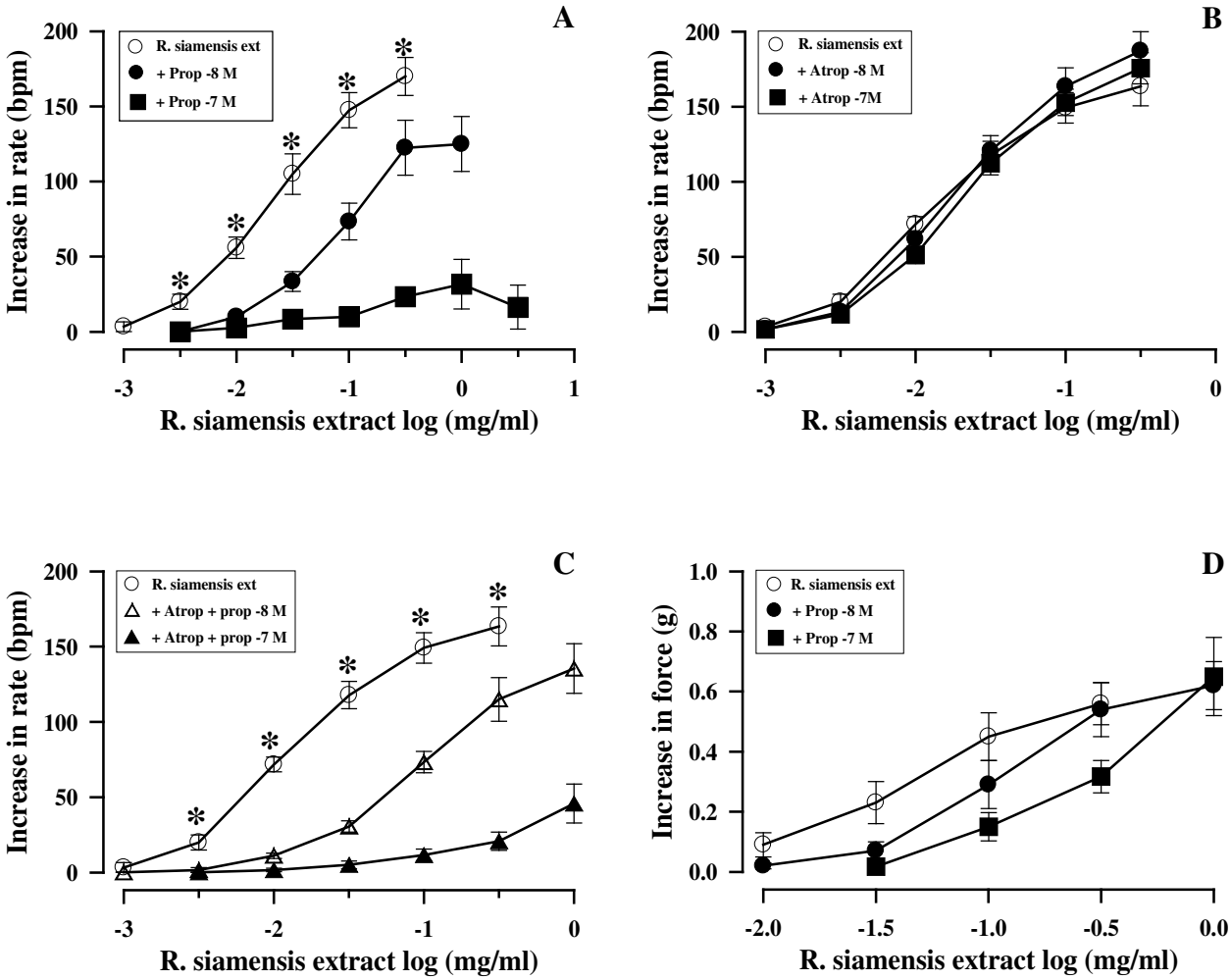
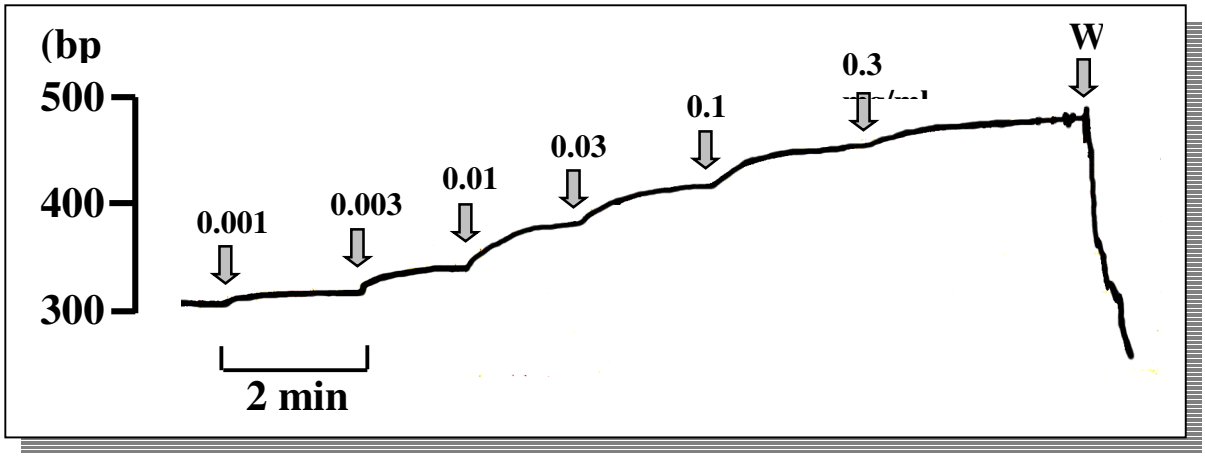


Figure 24 Effects of propranolol (prop, A, C and D) and/or atropine (atrop, B and C) on the positive chronotropic effects of spontaneous contraction of the right atrium (A-C) or on the positive inotropic effects of the electrical field-stimulated contraction of the left atrium (D) to *R. siamensis* extract. Each point represents mean ± S.E.M. of 6 experiments. * Significantly higher than those in the presence of propranolol and/or atropine, $p < 0.05$.

A



B

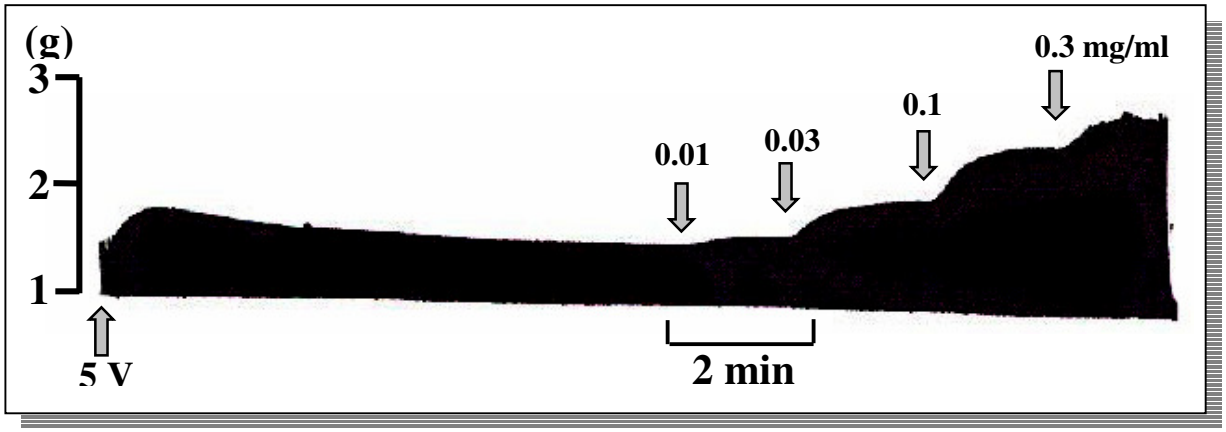


Figure 25 Typical recording showing effects of the *R. siamensis* extract on the increase in the rate of spontaneous contraction of the right atrium (A) and the increase in the strength of the electrical field-stimulated contraction of the left atrium (B). ⇩ indicates drug added in mg/ml of the final organ bath concentration. At w the bath solution was exchanged by washing with Krebs solution.

2.5.5 Effects of reserpine on the positive chronotropic and positive inotropic effects *of R. siamensis in isolated right and left atria of reserpinized rats*

In reserpine pretreated rats, the basal rate of spontaneous contraction of the right atrium are 243.33 ± 12.82 bpm, which is significantly lower than that of control group (291.67 ± 10.14 bpm). Whereas the basal force of electrical field-stimulated contraction of the left atrium are 0.37 ± 0.05 g, which is significantly bigger than that of the control group (0.24 ± 0.04 g).

A cumulative addition of the *R. siamensis* extract (0.001-0.3 mg/ml) caused an increase in both the rate of spontaneous contraction of the right atrium and the strength of the electrical field-stimulated contraction of the left atrium in a concentration-dependent manner. Pretreatment of rats with reserpine, both of these effects of the *R. siamensis* extract were suppressed (Figure 26).

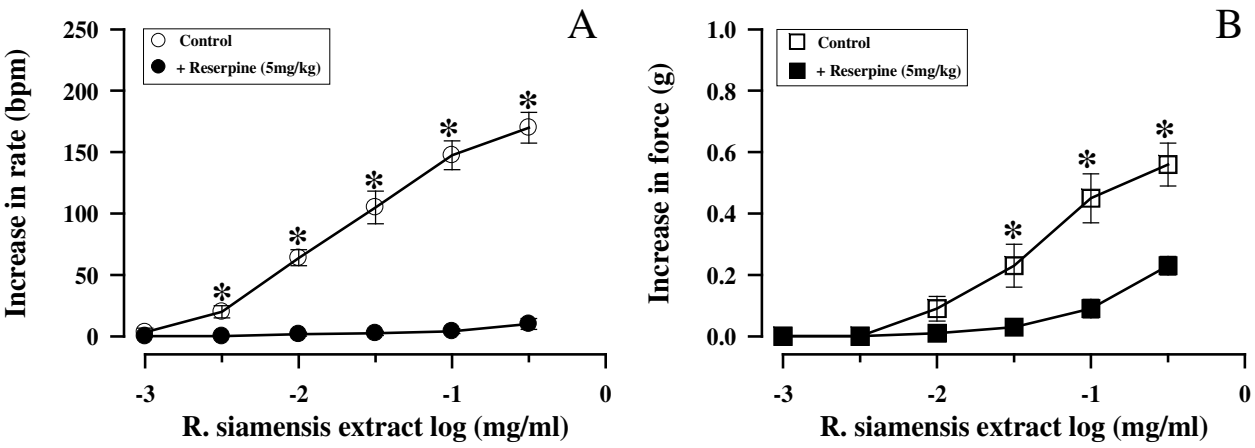


Figure 26 Effects of the depletion of the sympathetic neurotransmitters by reserpine on the positive chronotropic effect of spontaneous contraction of the right atrium (A) or on the positive inotropic effect of the electrical field-stimulated contraction of the left atrium (B) to *R. siamensis* extract. Each point represents mean ± S.E.M.

of 6 experiments. * Significantly higher than those of reserpinized tissues, $p < 0.05$.

2.5.6 Effects of the *R. siamensis* extract and phenylephrine on thoracic aortae *in vitro*

Typical recording of the vasoconstrictor responses to *R. siamensis* extract on the thoracic aorta is shown in Figure 28.

R. siamensis extract caused a slight increase of vasoconstriction of the endothelium-intact thoracic aortic rings. When the vascular endothelium was removed, or nitric oxide synthase was blocked by LNA, the vasoconstriction responses of the thoracic aorta to the *R. siamensis* extract were significantly increased (Figure 27A). However, removal of the endothelium caused a bigger response than did treatment with LNA. These effects were significantly inhibited by either phentolamine (Figure 27B), or reserpine treatment (Figure 27C).

Phenylephrine caused a concentration-dependent increase in vasoconstriction of the endothelium-intact thoracic aortic rings which were potentiated by pre-incubation of the blood vessels with LNA. When the rats were pretreated with reserpine, the contractile responses of the thoracic aortic rings to phenylephrine were enhanced for both the endothelium-intact and LNA-pretreated blood vessels (Figure 27D).

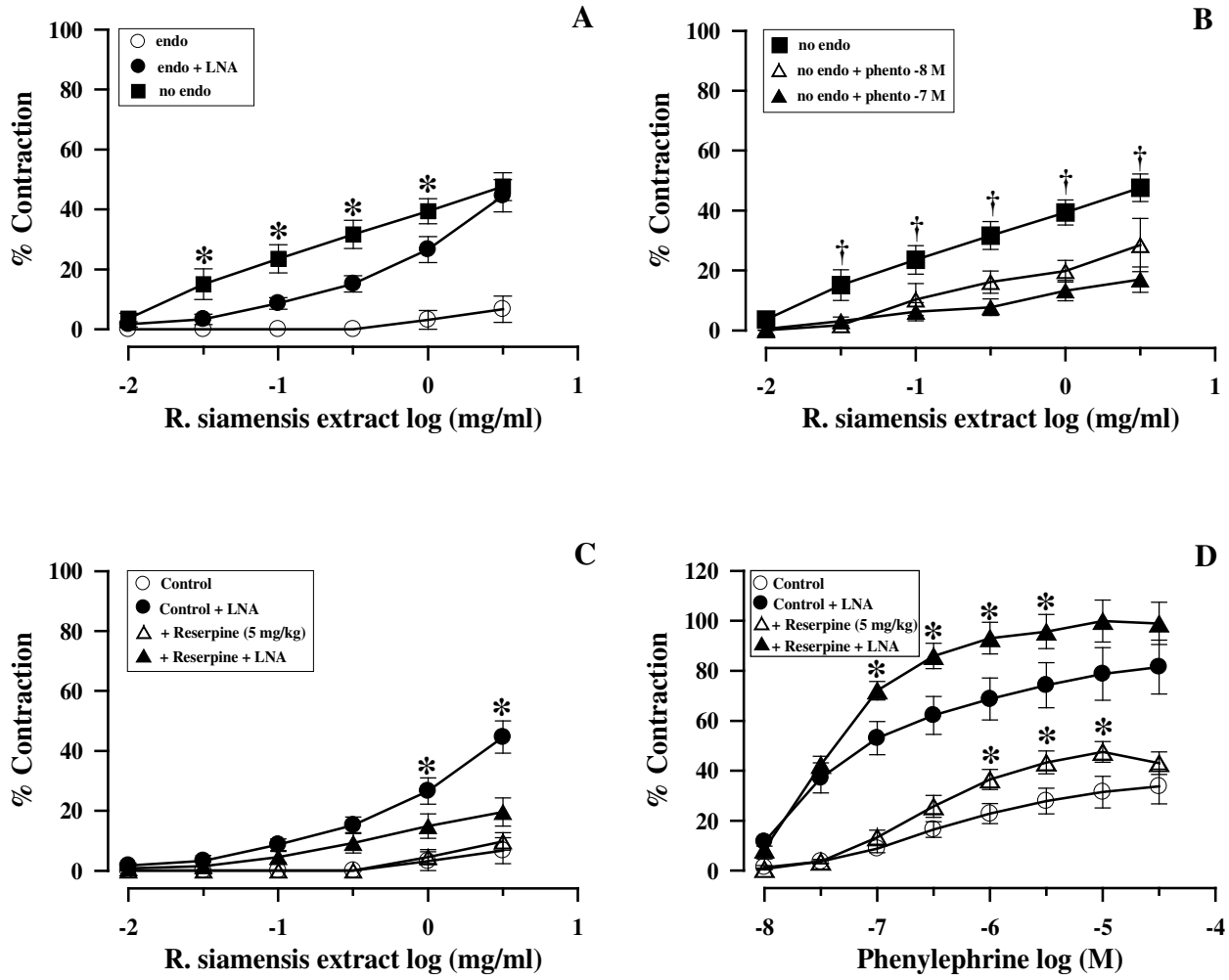


Figure 27 Effects of N^G-nitro-L-arginine (LNA, $3x10^{-4}$ M) or removal of vascular endothelium (A), phentolamine (B), or LNA and/or reserpine induced depletion of the sympathetic neurotransmitters (C and D) on the contractile response of the thoracic aortic rings to *R. siamensis* extract (A-C) or phenylephrine (D). Each point represents mean ± S.E.M. of 6 experiments. * Significantly higher than the ones with endothelium, or with LNA (A and D), or the other three groups (C), $p < 0.05$. † Significantly higher than the ones in the presence of phentolamine (B), $p < 0.05$.

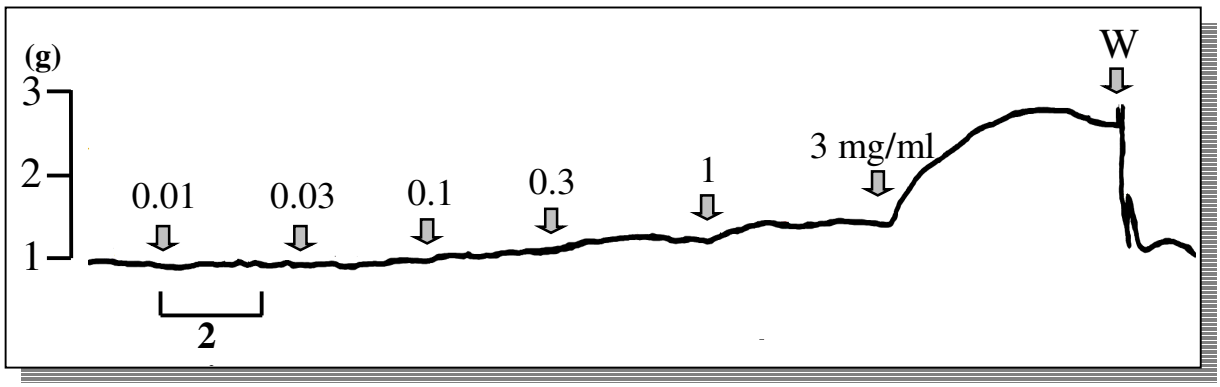


Figure 28 Typical recording showing a cumulative addition of the *R. siamensis* extract (0.01-3 mg/ml) increased force of contraction of the endothelium-intact thoracic aortic ring pre-incubated with $3x10^{-4}$ M LNA. ⇩ indicates drug added in mg/ml of the final organ bath concentration. At w the bath solution was exchanged by washing with Krebs solution.

2.5.7 Vasodilator activity of *R. siamensis* extract on rat thoracic aortae

Typical recording of the vasodilator responses to *R. siamensis* extract is shown in Figure 30.

R. siamensis extract (0.01-3 mg/ml) caused relaxation of the endothelium-intact thoracic aortic rings preconstricted with phenylephrine ($3x10^{-6}$ M). The sensitivity of the *R. siamensis* extract induced relaxation was increase in reserpinized rats. However, this effect disappeared after removal of the vascular endothelium, or after blocking the nitric oxide synthase by LNA (Figure 29).

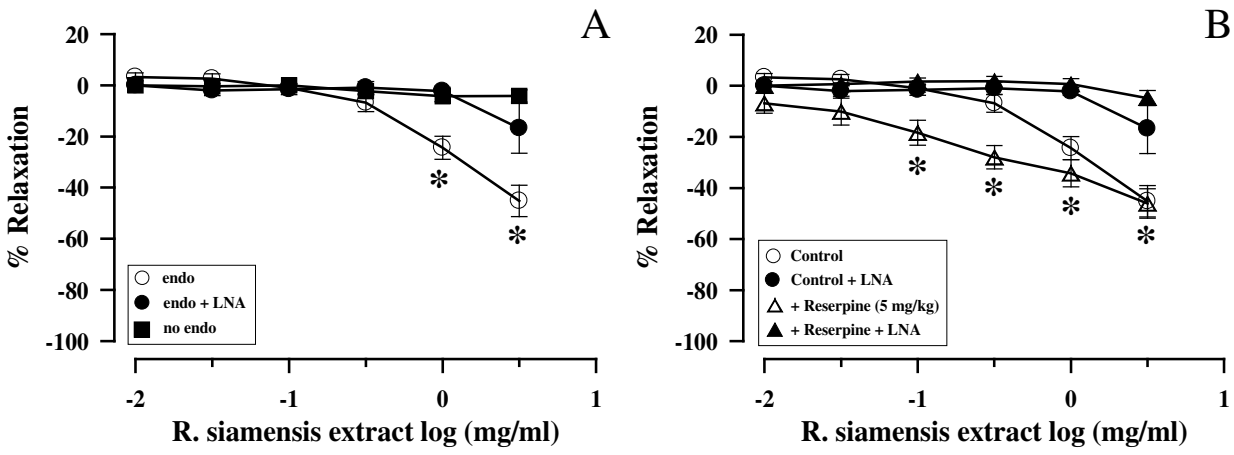


Figure 29 Effects of $3x10^{-4}$ M LNA, removal of vascular endothelium (A), and/or the depletion of the sympathetic neurotransmitters by reserpine (B) on the dilator response (preconstricted with $3x10^{-6}$ M phenylephrine) of the thoracic aortic rings to *R. siamensis* extract. Each point represents mean ± S.E.M. of 6 experiments. * Significantly lower than those without endothelium, with LNA, or control groups, $p < 0.05$.

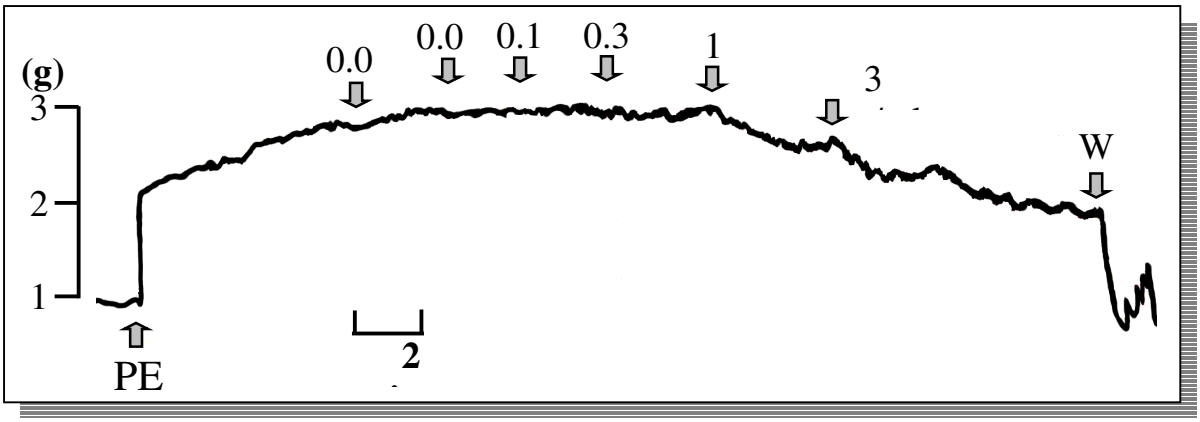


Figure 30 Typical recording showing a dose-dependent relaxation of an endothelium-intact thoracic aortic ring, preconstricted with $3x10^{-6}$ M phenylephrine (PE), to the *R. siamensis* extract (0.01-3 mg/ml). ⇩ indicates drug added in mg/ml of the final organ bath concentration. At w the bath solution was exchanged by washing with Krebs solution.

2.6 Discussion

The present study demonstrates that an *R. siamensis* extract can exert both a hypertensive and tachycardiac activity in anesthetized rats. These effects are similar to those produced by epinephrine, a non-specific α- and □-adrenergic receptor agonist (Figure 20), but not by norepinephrine, a non-specific α-adrenergic receptor agonist with lower □-adrenergic receptor activity, or isoproterenol, a non-specific □-adrenergic receptor agonist (Figure 19). However, small doses of the *R. siamensis* extract did not elicit hypotension, a property of epinephrine. This may be due to the absence of $\square_2$-adrenoceptor activity in the *R. siamensis* extract so the dilatation of the blood vessels produced by a small dose of epinephrine (Zhao et al., 2006) does not occur. However, this finding does indicate that the *R. siamensis* extract may act via both the α- and □-adrenergic receptors of the cardiovascular system. Further experiments to test this possibility were carried out in rats treated with propranolol, a non-specific □-adrenergic receptor antagonist and/or phentolamine, a non-specific α-adrenergic receptor antagonist (Brittain and Levy, 1976) before determining the dose-response curve to the *R. siamensis* extract and comparing it with epinephrine. The dose-response curve to the *R. siamensis* extract on MAP was not modified by 0.6 mg/kg propranolol. However 2 mg/kg phentolamine, that caused a rightward shift of the dose-response curve of norepinephrine and epinephrine, not only blocked the hypertensive response, but also caused a hypotension (Figure 21C). A similar result was found, when the animals were pretreated with both propranolol and phentolamine (Figure 21E). These results indicate that the *R. siamensis* extract most probably acts via the α-adrenergic receptors of the vascular system to cause hypertension, whereas it may act through another pathway of the vascular system to cause vasodilatation and hypotension. It is unlikely that the $\square_2$-adrenergic receptors of the blood vessels play a role in this activity since this effect was not significantly inhibited by propranolol.

Propranolol caused a rightward shift of the dose-response curve of the positive chronotropic effect of the *R. siamensis* extract (Figure 21B), and phentolamine also inhibited this chronotropic effect (Figure 21D). However, when the rat was pretreated with both propranolol and phentolamine, the dose-response curve to *R. siamensis* extract there was no additive effect (Figure 21F). A similar result was also found for epinephrine (Figure 20B and D). This suggests

that the chronotropic effect of α- and □-adrenergic receptor activation is being mediated by similar intracellular pathways, but further investigation is needed to clarify this. In any event the results show that even though there are far fewer α-adrenergic receptors compared to □-adrenergic receptor (Li et al., 1997; Bristow et al., 1988; Brodde and Leineweber, 2004), the positive chronotropic effect of the *R. siamensis* extract is mediated via both □- and α-adrenergic receptors.

The positive chronotropic and the hypertensive effects of the *R. siamensis* extract may be due to the active component(s) of the *R. siamensis* extract acting directly on the □- and α-adrenergic receptors or indirectly by stimulating the release of the sympathetic catecholamines. To examine this possibility, the rats were pretreated with reserpine to deplete stores of norepinephrine at the sympathetic nerve terminal and epinephrine at the adrenal medulla (Temma et al., 1977; Taesotikul et al., 1998; Weiner, 1985). The absence of norepinephrine and epinephrine in these reserpinized animals would reduce or abolish the positive chronotropic and hypertensive effects of the *R. siamensis* extract, if the active component acted indirectly by stimulating the release of the catecholamines. As shown in Figure 23, in reserpinized rats, the positive chronotropic and hypertensive effects of the *R. siamensis* extract disappeared and in addition, hypotension instead of hypertension occurred. These results demonstrate that the positive chronotropic and hypertensive effects of the *R. siamensis* extract on the cardiovascular system is an indirect action occurring via the release of the catecholamines, norepinephrine and epinephrine, non-specific adrenergic receptors, at the sympathetic nerve ending and at the adrenal medulla. The finding that *R. siamensis* extract caused hypotension in reserpinized rats, also confirms that the *R. siamensis* extract contains some substances other than those that cause vasodilatation.

The effect of the *R. siamensis* extract on the cardiovascular system in the *in vivo* experiments indicated that a substance in the extract may act peripherally to the cardiovascular tissues, or centrally through the central nervous system. In order to test whether it acts peripherally, further studies were performed *in vitro* on isolated preparations of atria and thoracic aortae. As shown in Figure 24, the *R. siamensis* extract caused an increase in both the rate of spontaneous contraction of the right atrium, and the strength of the electrical field-stimulated contraction of the left atrium in a concentration-dependent manner. These effects were blocked by

propranolol (10^{-8} and 10^{-7} M). However, the maximal response to *R. siamensis* extract in the presence of propranolol was significantly reduced (Figure 24A). Atropine, a non-specific muscarinic receptor antagonist, did not modify the positive chronotropic activity of the *R. siamensis* extract, because when it was added together with propranolol, no significant suppression was found for the chronotropic response of the *R. siamensis* extract (Figure 24C). These results indicate that the *R. siamensis* extract did not contain any other active component that acts via the muscarinic receptors of the atrium. The finding that the positive inotropic effect of the *R. siamensis* extract on the left atrium was also blocked by propranolol (Figure 24D). This result also confirmed that a component of the *R. siamensis* extract acts peripherally on the □-adrenergic receptors of the atrium.

In the previous *in vivo* experiment when rats had been pretreated with reserpine, two days before the experiments, the hypertensive and tachycardiac activities of the *R. siamensis* extract were abolished. This result indicates an indirect effect of the *R. siamensis* extract on the stimulated release of the sympathetic catecholamine from the sympathetic terminals and the adrenal medulla. In order to confirm this effect on the sympathetic nerve terminals, the concentration-response curve to the *R. siamensis* extract on isolated atria was studied using the atria from the rat having been pretreated with reserpine. In this case, the positive chronotropic and inotropic effects of the *R. siamensis* extract on isolated atria almost disappeared (Figure 26). These results confirm that the positive chronotropic and inotropic effects of the *R. siamensis* extract are likely to be an indirect effect by stimulating the release of the sympathetic neurotransmitters: norepinephrine from sympathetic nerve terminals to act via the □-adrenergic receptors of the atria.

The *R. siamensis* extract caused a contraction of the isolated thoracic aortic rings (Figure 27). This indicates that the hypertensive effect of the *R. siamensis* extract, at least partly, has a direct effect on the blood vessels. In addition, the constrictor responses to the *R. siamensis* extract were potentiated by blocking the nitric oxide synthase with LNA (Gross et al., 1990; Ishii et al., 1990; Moore et al., 1990), or by removal of the vascular endothelium. This result indicates that the *R. siamensis* extract may also stimulate release of nitric oxide from the vascular endothelium to attenuate its vasoconstrictor activity (Furchgott and Zawadzki, 1980). Furthermore, even though the rat thoracic aorta is poorly innervated (Nilsson, 1984; Nilsson,

1985; Nilsson et al., 1986) when the rat was pretreated with reserpine the constrictor responses of the aorta to phenylephrine were enhanced, whether LNA was presence or not (Figure 27D). This suggests that the receptor is under substantial influence from the sympathetic innervation, and that catecholamine depletion may cause receptor upregulation (Chess-Williams et al., 1987). Thus, the finding that the vasoconstriction response of the *R. siamensis* extract could be suppressed by phentolamine, or by pretreating the rat with reserpine, also indicated that the *R. siamensis* extract may act indirectly via the α-adrenergic receptor in the blood vessels by stimulating release of norepinephrine.

The *R. siamensis* extract also caused a decrease in the mean arterial blood pressure in anesthetized rats after blocking the α- and □-adrenergic receptors with phentolamine and propranolol respectively. This may be caused by a direct effect of the *R. siamensis* extract on the blood vessels inducing vasodilatation. To test this possibility, we did experiments on the vasodilatation of the *R. siamensis* extract on thoracic aortic rings preconstricted with phenylephrine. As shown in Figure 29, the *R. siamensis* extract induced a concentration-dependent vasodilatation. However, the vasodilatory activity of the *R. siamensis* extract may be a direct effect of the active component on the vascular smooth muscle, or via an indirect pathway that stimulated the release of the nitric oxide from the endothelium and caused vasodilatation. To test these possibilities, further studies were carried out in denuded-thoracic aortic rings, as well as on endothelium-intact aortic rings in the presence of LNA. It was found that the vasodilatory activity of the *R. siamensis* extract on the thoracic aorta was abolished after removal of the vascular endothelium or by blocking the nitric oxide synthase with LNA. In addition, when the thoracic aortic rings have undergone a depletion of their sympathethetic neurotransmitters by pretreating the rats with reserpine, two days before the experiments, there was an enhanced sensitivity of the vasodilatory effect of the *R. siamensis* extract (Figure 29B). These results indicate that the vasodilatory activity of the *R. siamensis* extract is an indirect effect of the substance acting by stimulating release of the nitric oxide from the vascular endothelium and causing vasodilatation. This effect was clearly shown when the contractile effect produced by a sympathetic neurotransmitter was abolished by pretreating the animal with reserpine that depletes the sympathetic neurotransmitter of norepinephrine. These results indicate that the hypotensive activity of the *R. siamensis* extract in anesthetized rats after blocking the adrenergic

receptors is a direct effect of the *R. siamensis* extract on the blood vessels causing vasodilatation by stimulating release of nitric oxide from the vascular endothelium.

These results differ from our previous report on the effect of the pseudoginsenoside-RT_1 on blood pressure and heart rate, when it was found that the pseudoginsenoside-RT_1 caused a decrease in the mean arterial blood pressure with an increase in heart rate in a dose-dependent manner similar to those produced by isoproterenol, a non-specific □-adrenergic receptor agonist. Whereas in the present study it was found that the *R. siamensis* extract caused an increase in both MAP and heart rate similar to that of epinephrine, whereas the hypotensive activity was found only when the rats were pretreated with phentolamine or phentolamine plus propranolol. This indicates that the positive chronotropic effect and the hypotensive effect are caused by different substances (Jansakul et al., 1999). The reason for these different results could be that the pseudoginsenoside-RT_1, isolated by Aukkanibutra (1985), was not 100 % pure, and may be contaminated with a positive chronotropic substance. As we know that *R. siamensis* extract contains more than one compound that affects blood pressure and heart rate the reason for these different results could be that the pseudoginsenoside-RT_1, isolated by Aukkanibutra (1985), had different proportions of the active substances than did the preparation we used in these studies.

2.7 Conclusion

The present study has demonstrated that *R. siamensis* extract has both a hypertensive and a positive chronotropic effects on the cardiovascular system, by stimulating the release of endogenous catecholamines, most likely from nerve endings and the adrenal medulla to act directly at the α- and □-adrenergic receptors of the heart and the blood vessels. It also has a hypotensive activity, acting directly on the blood vessels to cause vasodilatation by stimulating the release of nitric oxide. The active substance(s) responsible for the hypertensive and positive chronotropic effects are likely to be different from those causing hypotension, since the hypotensive activity persisted after having depleted the sympathetic neuronal transmitters by reserpine. These findings provide scientific support for the traditional uses of the plant in man: e.g. induced abortion which would be caused by stimulated release of endogenous catecholamine

producing uterine contraction, and for controlling blood pressure which would be caused by stimulated release of the nitric oxide from vascular endothelium. However, further study is required to identify the active substance(s) responsible for these activities. This could then provide an opportunity to develop new chemically pure cardiovascular drugs with known properties and further confirm the beneficial effects of the Thai therapeutic uses of this plant.