CHAPTER 3

BIOACTIVE CONSTITUENTS FROM FRESH FRUITS

OF *RANDIA SIAMENSIS*

3.1 Abstract

Bioactive-directed fractionation of n-butanol extract from fresh fruits of *Randia siamensis* led to isolate of 4 new pseudoginsenoside compounds: pseudoginsenosides-RT_2, -RT_3, -RT_4 and -RT_5, together with 7 known compounds: tyramine, pseudoginsenosides-RT_1 methyl ester, pseudoginsenosides-RT_1, pseudoginsenoside-RP_1, kaempferol-3-O-β-D-xylose(1-2)-□β-galactose, kaempferol-3-O-β-D-galactose, and 5-O-[Z] caffeoylquinic acid. Structures of the compounds were analyzed by NMR, MS and GC. Tyramine is responsible for the hypertensive activity, whereas pseudoginsenosides-RT_1 and pseudoginsenoside-RP_1 are responsible for the hypotensive activity.

3.2 Introduction

In chapter 2 it was found that n-butanol extract from fresh fruits of *R. siamensis* (*Randia siamensis* extract) produced a hypertensive and a positive chronotropic effects in anesthetized rats. It also produced a hypotensive activity, if the animals were pretreated with phentolamine, a non-specific adrenergic receptor antagonist, which indicated that at least three different activities were found in the n-butanol extract from fresh fruits of the *R. siamensis*. Thus it is of interest to isolate the pure compounds and find out whether those activities are responsible by one compound or one for each activity. The knowledge obtained would be useful for folkloric therapy, as well as provided an opportunity to develop the pure or partial pure compounds as a cardiovascular drug.

3.3 Objectives

To identify the active substance(s) responsible for the hypertensive and positive chronotropic effects as well as the hypotensive effects from crude n-butanol extract from fresh fruits of *Randia siamensis*.

3.4 Materials and Methods

3.4.1 Extraction and Isolation

The n-butanol extract from fresh fruits of *Randia siamensis* (or *R. siamensis* extract) was prepared using the same method as that described in Chapter 2.

Isolation of active substance (s) from the *R. siamensis* extract is followed diagram in Figure 31. The *R. siamensis* extract (50 g) was subjected to column chromatography over silica gel 100 (850 g) and eluted with the mixture solvent of $CHCl_3$: MeOH : H_2O (65:35:7, 3 L and 60:40:8, 1.5 L) and 100% MeOH (1 L). The eluted solution was collected at the volume of 70 ml/tube. Twelve fractions, which were combined into 5 fractions (A1-A5), were obtained. Fraction A2 (15.38 g) was re-subjected to column chromatography (silica gel 60; 230-400 □m, 850 g), eluted with $CHCl_3$: MeOH : H_2O (65:35:5, 4 L and 60:40:8, 1 L) affording 4 major fractions (B1-B4). Fraction B2 (791.42 mg), was separated using column chromatography over sephadex G-15 (500 g) and a mixture of MeOH : H_2O (1:1, 0.5 L) as an eluent. The eluted solution was collected at the volume of 10 ml/tube. Fraction C4 afforded **compound 1** (**RS1,** 23.6 mg) and fraction C5 afforded **compound 2** (**RS2**, 27.1 mg). The cardiovascular active fraction A3 (17.56 g), which was also obtained from silica gel 100 column, was subjected to column chromatography over silica gel 60 (850 g) and eluted with $CHCl_3$: MeOH : H_2O (65:35:5, 4 L and 60:40:8, 1 L) affording 5 fractions (E1-E5). The fraction E2 (2.09 g), which showed a slightly decrease in MAP in anesthetized rats, was further fractionated by silica gel reverse phase C_{18} column using gradient elution of MeOH : H_2O; 50% MeOH (2.5 L), 60% MeOH (6 L) and 70% MeOH (2.8 L). The fractions was collected at the volume of 17 ml/tube, 8 fractions (F1-F8) were

obtained, fraction F5 afforded **compound 3** (**RS3**, 535.5 mg). Fraction F6 (194.8 mg) was purified by semi-preparative HPLC using isocratic elution (70% MeOH + 0.05% TFA) on SymmetryPrep™ C_{18} column (7 □m, 19 x 150 mm; Waters, flow rate 10 ml/min, detection UV 210 nm), yielding **compound 4** (**RS4**, 26.8 mg). The active fraction E3 (8.89 g), which induced increases in MAP and heart rate in anesthetized rats, was fractionated by silica gel reverse phase C_{18} (600 g) column using gradient elution of MeOH : H_2O; 40% MeOH (1.5 L), 50% MeOH (1.5 L), 60% MeOH (1.5 L), 70%MeOH (2.5 L) and 80% MeOH (1 L). The solution was collected at the volume of 17 ml/tube. Thirty-five fractions, which were combined into 8 fractions (H1-H8), were obtained. Fraction H5 (2.36 g) was separated by semi-preparative HPLC (semi-prep. HPLC) with MeOH-H_2O-0.05% TFA on a □Bondapak® C_{18} pre-packed column (10 □m, 25x100 mm, Waters, isocratic: MeOH 35%, flow rate 10 ml/min, UV 210 nm), yielding **compounds 5** (**RS5**, 259.1 mg) and **6** (**RS6**, 945.9 mg). Fraction A4 (1.12 g), which was obtained from silica gel 100 column, was separated by silica gel reverse phase C_{18} column using the gradient solution of MeOH : H_2O; 20% MeOH (6.5 L), 30% MeOH (6 L), 40% MeOH (5 L), 50% MeOH (5.5 L), 60% MeOH (3 L) and 70% MeOH (2 L), 17 fractions were obtained and combined into 5 fractions (K1-K5). Fraction K2 and K4 yields **compounds 7 (RS7**, 270.6 mg) and **8 (RS8**, 299.9 mg), respectively. Fraction K1 (70.2 mg) was separated by semi-preparative HPLC with MeOH-H_2O-0.05% TFA on a □Bondapak® C_{18} pre-packed column (10 □m, 25x100 mm, Waters, isocratic: MeOH 10%, flow rate 10 ml/min, UV 210 nm), yielding **compound 9 (RS9**, 32.5 mg). Fraction K3 (120.8 mg) and fraction K5 (215.1 mg) were also purified by semi-preparative HPLC with MeOH-H_2O-0.05% TFA on a SymmetryPrep™ C_{18} column (7 □m, 19 x 150 mm; Waters, isocratic: MeOH 45% and 50%, flow rate 10 ml/min, UV 210 nm), yielding **compounds 10 (RS10**, 22.4 mg) and **11 (RS11**, 18 mg).

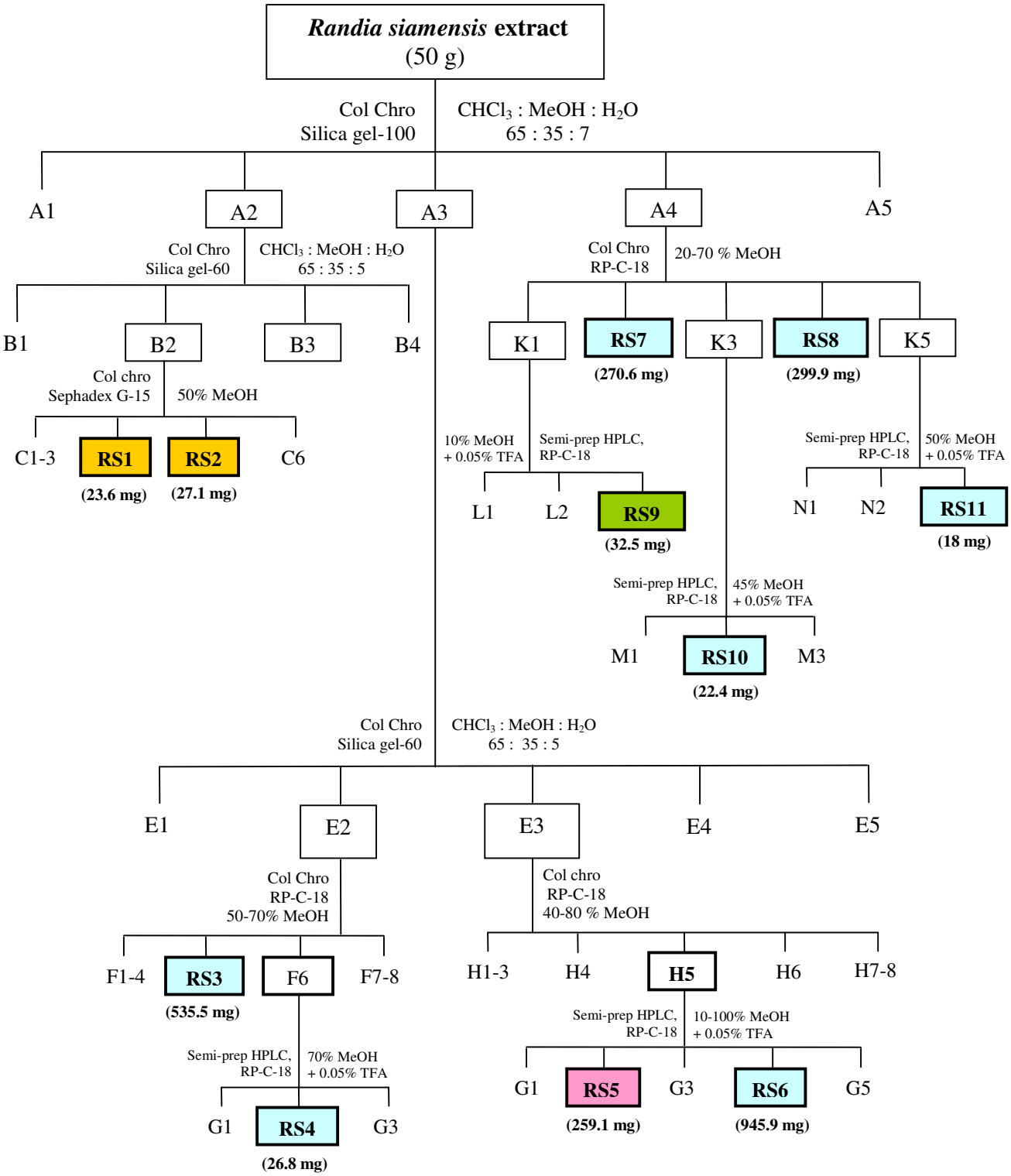


Figure 31 Flow chart showing isolation of constituents from

3.4.2 Structural determination

Chemical structures of the compounds isolated from the *R. siamensis* extract were elucidated by Prof. Kurt Hostettmann and his colleges, University of Geneva, Switzerland, using spectroscopic and chemical methods**.**

General experimental procedures

UV spectra were measured on a Perkin-Elmer Lambda 20 spectrophotometer (Perkin-Elmer, Boston, MA USA). GC-MS was performed in a TSQ70 Varian (Palo Alto, CA, USA) using a capillary column SPB-50 (30 m x 0.25 mm i.d., Supelco). HRESIMS were recorded on a Brunker FTMS 4.7 T mass spectrometer (Brunker, Billerica, MA). EIMS spectra were obtained on a Finnigan-MAT/TSQ-700 triple stage quadrupole instrument (EIMS: 70 eV). Mass spectra were obtained on a Finnigan-MAT/TSQ-700 triple stage quadrupole instrument (Finningan MAT, San Jose, CA, USA). ^{1}H and ^{13}C NMR were recorded on a Varian Inova 500 spectrometer (Varian, Palo Alto, CA, USA) (500 MHz and 125 MHz, respectively) in DMSO-d_6 or $CDCl_3$; chemical shifts in ppm as □□rel. to Me_4Si (internal standard). TLC was performed using silica gel 60 F_{254} Al sheets (Merck), detection at 210 and 254 nm. Analytical HPLC was carried out on a HP 1100 system equipped with a photodiode array detector (Agilent technologies, Wilmington, DE, USA). Extracts and fractions were analyzed on a SymmetryShield C_{18} column (5 □m, 2.1 x 150 mm; Waters, Bedford, MA, USA). Semi-preparative HPLC was carried out with a Shimadzu LC-8A pump (Shimadzu, Kyoto, Japan) equipped with a Knauer UV detector using a □Bondapak® C_{18} prepacked column (10 □m, 25 x 100 mm; Waters) and SymmetryPrep™ C_{18} column (7 □m, 19 x 150 mm; Waters), flow rate 10 ml/min, detection UV 210 nm.

Sugar hydrolysis

The hydrolysis was performed on compounds **1-4** and **6-8** to determine the nature of the sugar moiety. The reaction was realized on 2 mg of each product. The compounds

were refluxed with a solution of HCl at 2N during 4 hrs. The solution was then neutralized with Na_2CO_3 and extracted with CH_2Cl_2 to give an aqueous fraction containing the sugar(s) and a CH_2Cl_2 fraction containing the aglycon.

Sugar analysis by GC-MS

The GC analysis was performed with the sylilated sugars. The sylilation reaction was performed using 0.5 mg of the fraction containing the sugar(s). The sugar fractions obtained by hydrolysis was soluble in a mixture of pyridine (0.2 ml) and BSTFA (N,O-bis(trimethylsilyl) trifluoracetamide) (0.2 ml). The mixture was stirred during 2 hrs after the solvents are evaporated by N_2 flow at room temperature. The crude reaction was soluble in 0.5 ml of heptane and 1 □l was analyzed by GC using the sugar standards. GC-MS were performed using a 30 m x 0.25 mm i.d. SPB-50 (Supleco) column connected with to a Finnigan-MAT/TSQ-700 triple stage quadrupole MS instrument, with an initial temperature 70^{o}C for 1 min and then temperature was programmed to 300^{o}C at a rate of 10^{o}C/min. The retention times and fragmentation patterns of the analytes were compared with those of authentic sugar sylilated standards.

3.4.3 Pharmacological studies of the isolated compounds

Effects on mean arterial blood pressure and heart rate *in vivo*

Female Wistar rats (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 access to standard food and tap water *ad libitum.* Preparation of animals followed the Prince of Songkla University guidelines for the approved 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.

After 40-min equilibration, the dose-response relationships to tyramine, pseudoginsenoside-RP_1, -RT_1, -RT_3 (new) and -RT_5 (new), the major constituents, were studied. Each rat was used for only one substance. Pseudoginsenoside-RP_1 was dissolved in 10 % DMSO (V:V in distilled water), and the rest of them were dissolved in distilled water.

Changes in blood pressure and heart rate were recognized as the difference between the steady pressure before and the lowest pressure after injection. The blood pressure were recorded in mmHg as systolic pressure (SP) and diastolic pressure (DP) and were expressed as mean arterial blood pressure (MAP), which was calculated as DP+1/3 (SP-DP). Results are expressed as mean ± S.E.M. of 6 experiments (n=6).

Effects on vasodilatation of isolated rat thoracic aortae and mesenteric arteries *in vitro*

Isolated rat thoracic aortae were prepared from female Wistar rats using the same method as that described in Chapter 2. After equilibration, the relaxing responses to pseudoginsenoside-RT_1 (10^{-7}-10^{-2} M) were investigated on endothelium-intact thoracic aortae preconstricted with $3x10^{-6}$ M phenylephrine.

Male Wistar rats (250-350 g), used for the *in vitro* preparation of small mesenteric artery, were supplied from the Animal House, Department of Pharmacology, University of Aarhus, Denmark. They were maintained in controlled environmental conditions (24-26^{o}C) with a 12 h light/dark cycle and access to standard food and tap water *ad libitum.*

Rats were killed by cervical dislocation. Mesenteric arteries were prepared from the rats according to Mulvany & Halpern (1977). Briefly, second- or third-order branches of the superior mesenteric artery (250-400 μm) were isolated from surrounding adipose and connective tissue, and 2-mm long segments of this vessel were removed. Each vessel segment was mounted as a ring preparation on two 40 μm stainless steel wires in a dual wire myograph chamber (Figure 32A and B) containing physiological salt solution (PSS, in mM: NaCl 119, KCl 4.7,

$CaCl_2$ 2.5, $MgSO_4$ 1.17, KH_2PO_4 0.4, $NaHCO_3$ 14.9 and glucose 5.5) at 37°C. One wire was connected to a force transducer for isometric recording of tension development and the other was attached to a micrometer. The myograph chamber was gassed continuously with 5% CO_2 and 95% O_2 to maintain a pH of 7.4. For the endothelium-denuded artery, the endothelium was removed by introducing a human hair into the lumen and rubbing it carefully backwards and forwards several times.

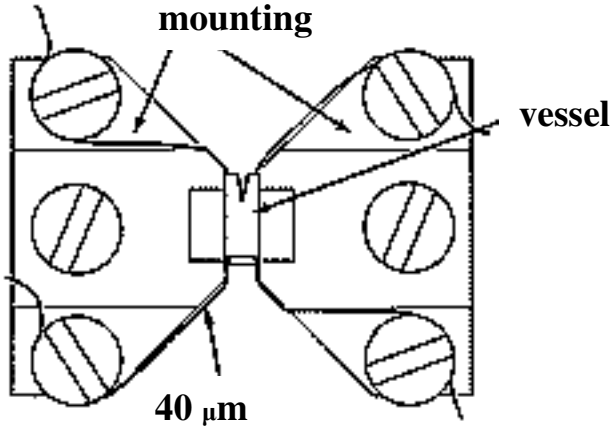
After 30-min equilibration under zero tension in PSS, the normalization was performed by distending the vessel stepwise and measuring sets of micrometer (x) and force readings (y). These data were converted into values of internal circumference (IC, □m) and wall tension T (mN/mm), respectively. However, the stepwise distension was continued until the calculated effective pressure exceeded the target transmural pressure which is normally 100 mmHg (T_{100} = 13.3 kPa) for rat mesenteric arteries. Plotting wall tension against internal circumference reveals an exponential curve and by applying the isobar curve corresponding to 100 mmHg. Then, the internal circumference corresponding to 100 mmHg (IC_{100}) and the normalized internal circumference (IC_1) were calculated. In addition, the normalized internal diameter l_1 is calculated by dividing IC_1 with ¶. Before the experiments, basal tension of the vessels was set to a normalized internal circumference of IC_1, where IC_1 is $0.9IC_{100}$.

After the normalization process and re-equilibration for 30 min with changes of PSS at a 10-min interval, the viability of vessels was tested using the standard procedure, which consisted of stimulating vessels three times with PSS containing 10^{-5} M norepinephrine. The vessels were activated for 2 min with each solution followed by a 5 min washout with PSS to allow full relaxation. Myograph-mounted vessels were considered viable if the effective active pressure developed to PSS containing norepinephrine was □13.3 kPa. The presence of functional endothelium of the vessels was assessed by the ability of 10^{-5} M acetylcholine to induce □80% relaxation of vessels preconstricted with 10^{-6} M phenylephrine. In the experiments of endothelium-denuded arteries, the absence of acetylcholine-induced relaxation was taken as an indicator of successful denudation.

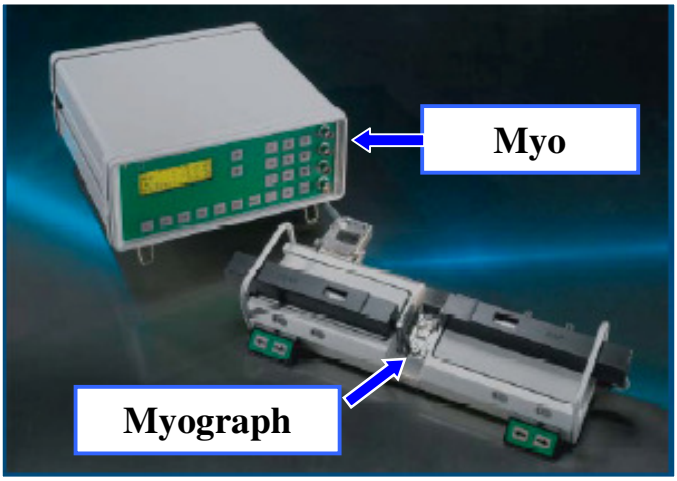
Before starting each experiment, the vessels were incubated with 10^{-6} M propranolol and $3x10^{-6}$ M cocaine to block β-adrenergic receptors and neuronal catecholamine uptake for 15 min. After that the concentration-response relationship to pseudoginsenoside-RT_1

(10^{-7}-10^{-2} M) or pseudoginsenoside-RP_1 (10^{-7}-10^{-3} M) on %relaxation was constructed both in endothelium-intact and -denuded mesenteric arteries, preconstricted with 10^{-6} M phenylephrine.

A



B



C

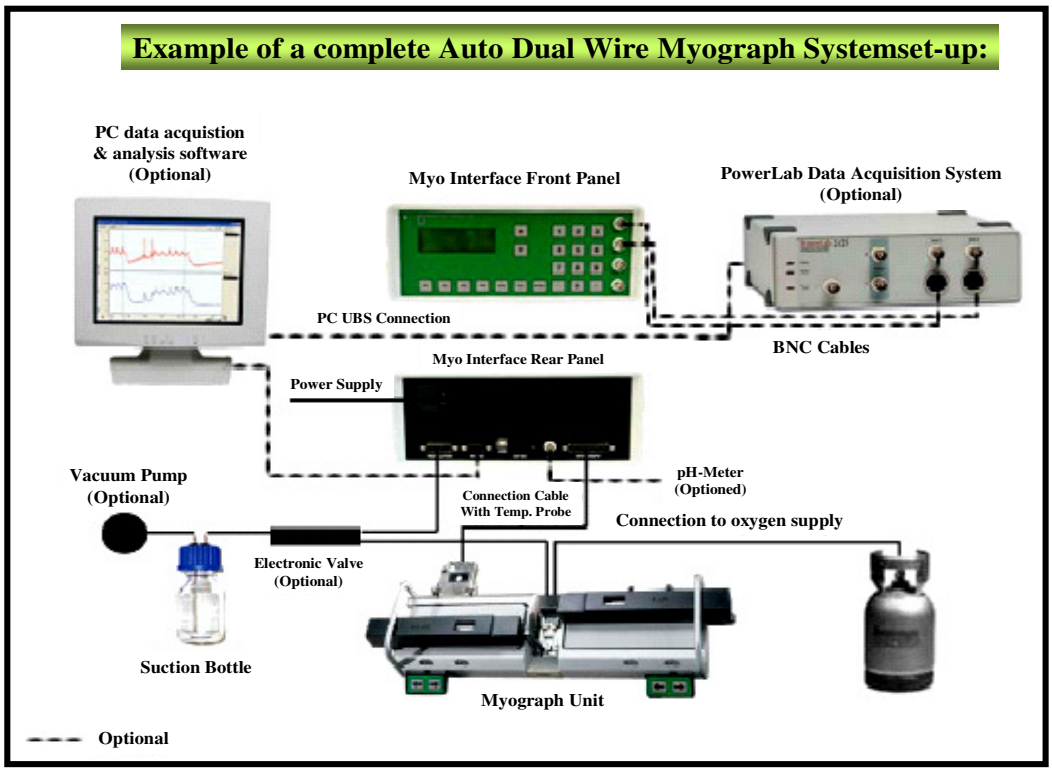


Figure 32 Illustration showing preparation of a vessel segment on two 40 μm stainless steel

wires (A), a dual wire myograph chamber (B) and auto dual wire myograph

3.4.4 Drugs and chemicals

The following drugs were used: propranolol hydrochloride, phentolamine hydrochloride, (-)- norepinephrine bitartrate, phenylephrine hydrochloride, acetylcholine and cocaine, they dissolved in a solution (g/L): NaCl 9.0, NaH_2PO_4 0.19 and ascorbic acid 0.03. All drugs were purchased from Sigma, U.S.A.

The organic solvents for isolation: methanol and chloroform, and the silica gel for column chromatography: silica gel 100 (70-230 mesh ASTH), silica gel 60 (230-400 mesh ASTH) and silica gel 60 RP-18 (40-63 □m), were purchased from Merck, Germany.

3.4.5 Data analysis and statistics

Data are expressed as mean □ S.E.M. of 6 experiments for *in vivo* study on anesthetized rats, and 3 experiments for *in vitro* study on isolated rat mesenteric artery. Vasodilator responses of mesenteric arteries were expressed as mean □ S.E.M. of percentage relaxation of vessels from 10^{-6} M phenylephrine preconstriction-levels. Test of significance were done using the Student's paired t-test. In all cases, a *p* value of 0.05 or less was considered statistically significant.

3.5 Results

3.5.1 Isolated compounds

As shown in Table 1 and Figure 33, 11 compounds were isolated from *Randia siamensis* extract are as follows:

Kaempferol-3-*O*-β-xylose (1-2)- β-galactoside (**1**): Yellow amorphous powder.

Kaempferol-3-*O*-β-galactoside (**2**): Yellow amorphous powder.

Pseudoginsenoside-RP$_1$ (**3**): White amorphous powder.

Pseudoginsenoside-RT$_1$ methyl ester (**4**): White amorphous powder.

Tyramine (**5**): White amorphous powder.

Pseudoginsenoside-RT$_1$ (**6**): White amorphous powder.

Pseudoginsenosides-RT$_5$ (**7**): White amorphous powder.

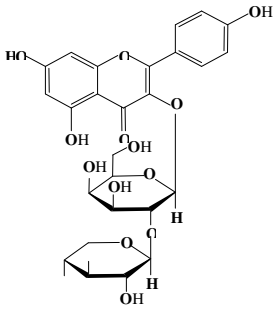
Pseudoginsenosides-RT$_3$ (8): White amorphous powder.

5-*O*-[Z] caffeoylquinic acid (**9**): Brown amorphous powder

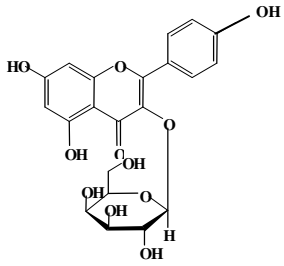
Pseudoginsenosides-RT$_4$ (10): White amorphous powder.

Pseudoginsenosides-RT$_2$ (**11**): White amorphous powder.

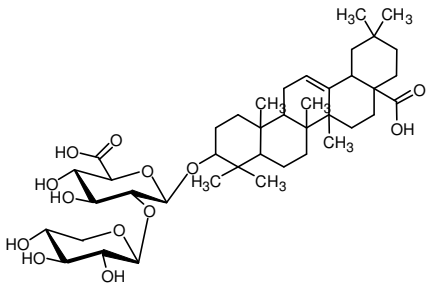
The chemical structures of the above substances were elucidated by Prof. Hostettman and his colleges (details in appendix).



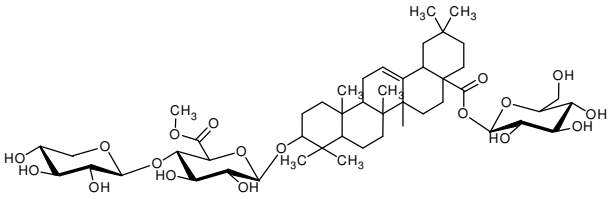
Kaempferol-3-*O*-ß-D-xylose (1-2)-ß-D-galactoside (compound 1, RS1)



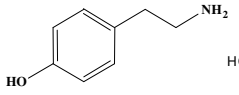
Kaempferol-3-*O*-ß-D-galactoside (compound 2, RS2)



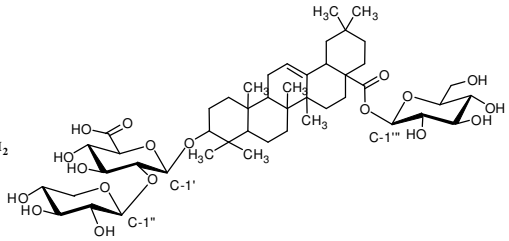
Pseudoginsenoside-RP$_1$ (compound 3, RS3)



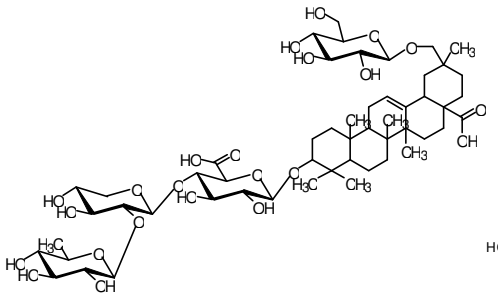
Pseudoginsenoside-RT$_1$ methyl ester (compound 4, RS4)



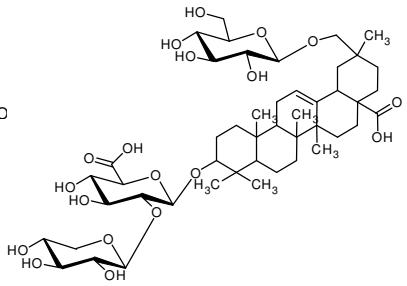
tyramine (compound 5, RS5)



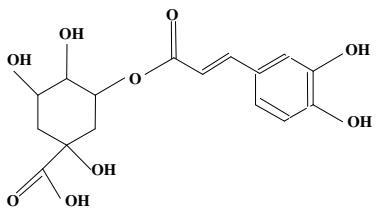
Pseudoginsenoside-RT$_1$ (compound 6, RS6)



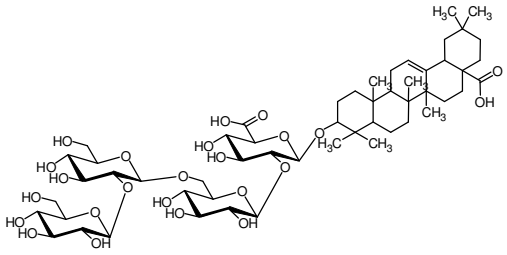
Pseudoginsenoside-RT$_5$ (compound 7, RS7)



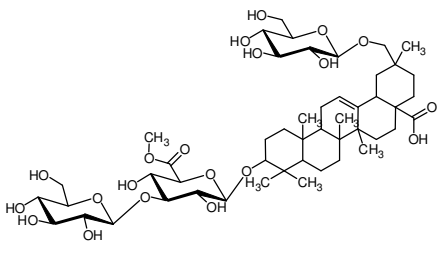
Pseudoginsenoside-RT$_3$ (compound 8, RS8)



5-*O*-[Z] caffeoylquinic acid (compound 9, RS9)



Pseudoginsenoside-RT$_4$ (compound 10, RS10)



Pseudoginsenoside-RT$_2$ (compound 11, RS11)

Figure 33 Structures of compounds isolated from the

3.5.2 Effects of tyramine, pseudoginsenoside-RT_1,-RT_3 and -RT_5 and pseudoginsenoside-RP_1 on the mean arterial blood pressure and heart rate in anesthetized rats

Typical recording of the effects on blood pressure and heart rate of tyramine, pseudoginsenoside-RT_1 and pseudoginsenoside-RP_1 are shown in Figure 34.

Intravenous injection of tyramine (0.01-1 mg/kg) caused dose-dependent increases in MAP and heart rate in anesthetized rats (Figure 35A and B). These effects last within 5 min for the highest dose at 1 mg/kg (Figure 34A). Low doses of pseudoginsenoside-RT_1 (1-3 mg/kg) produced minute and transient decrease in MAP with no changes in heart rate. High doses of the pseudoginsenoside-RT_1 (10 and 30 mg/kg) caused substantial decreases in MAP with slight decreases in heart rate (Figure 35C and D) and this effect last over 60 min (Figure 34B). In case of pseudoginsenoside-RP_1, a similar result as that of pseudoginsenoside-RT_1 was obtained when small doses were given into the animals (Figure 35E and F). However, at high doses (10 and 30 mg/kg), pseudoginsenoside-RT_1 produced a transient decrease in MAP with prolong (over 30 min) decrease in heart rate were obtained (Figure 34C). The other two major components, pseudoginsenoside-RT_3 and -RT_5, at the doses of 10 and 30 mg/kg, produced no changes in MAP and heart rate in anesthetized rats (data not shown).

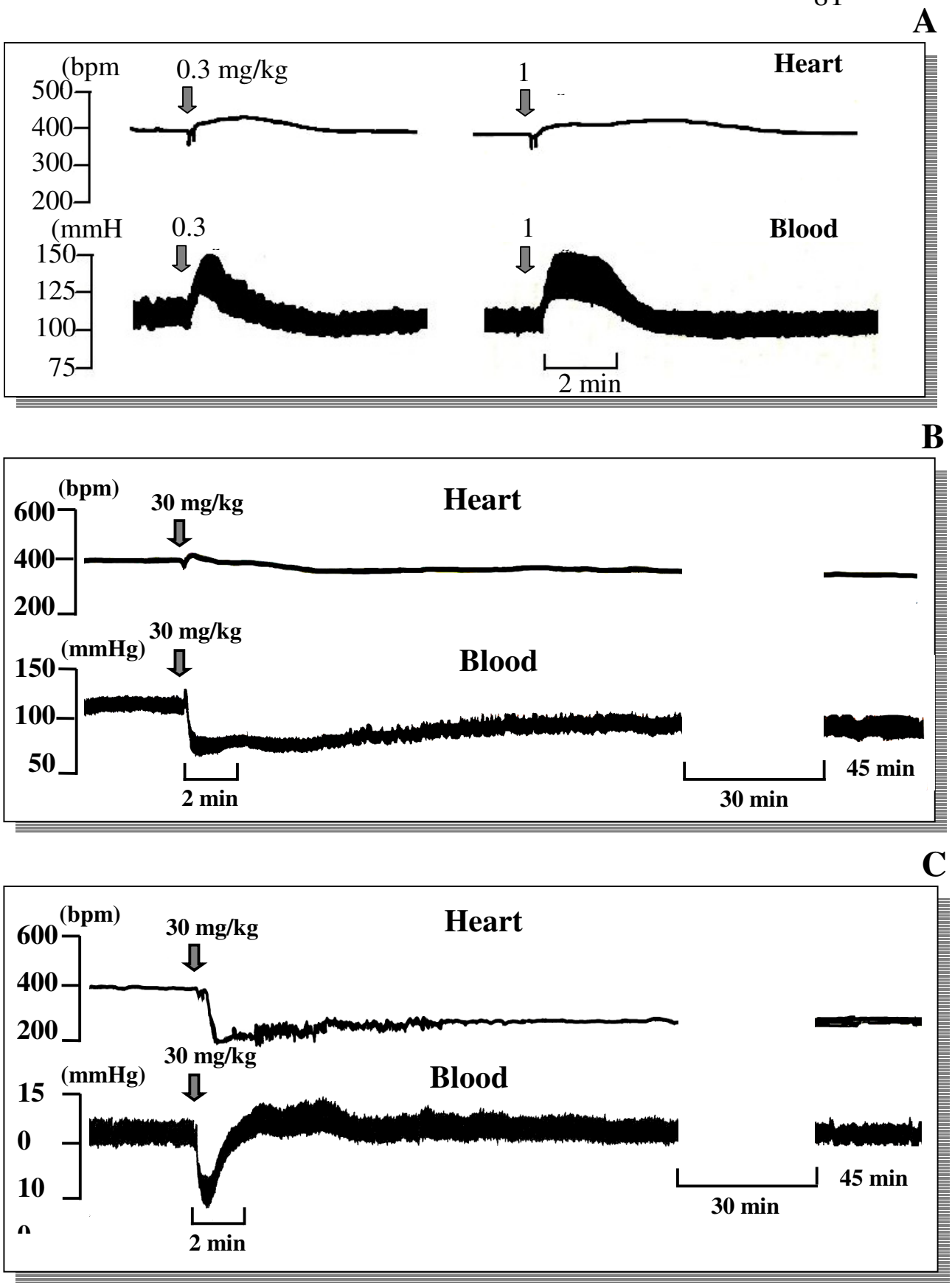


Figure 34 Typical recording showing effects of intravenous injections of tyramine (0.3 and 1 mg/kg,

A), pseudoginsenoside-RT_1 (30 mg/kg, B) and pseudoginsenoside-RP_1 (30 mg/kg, C)

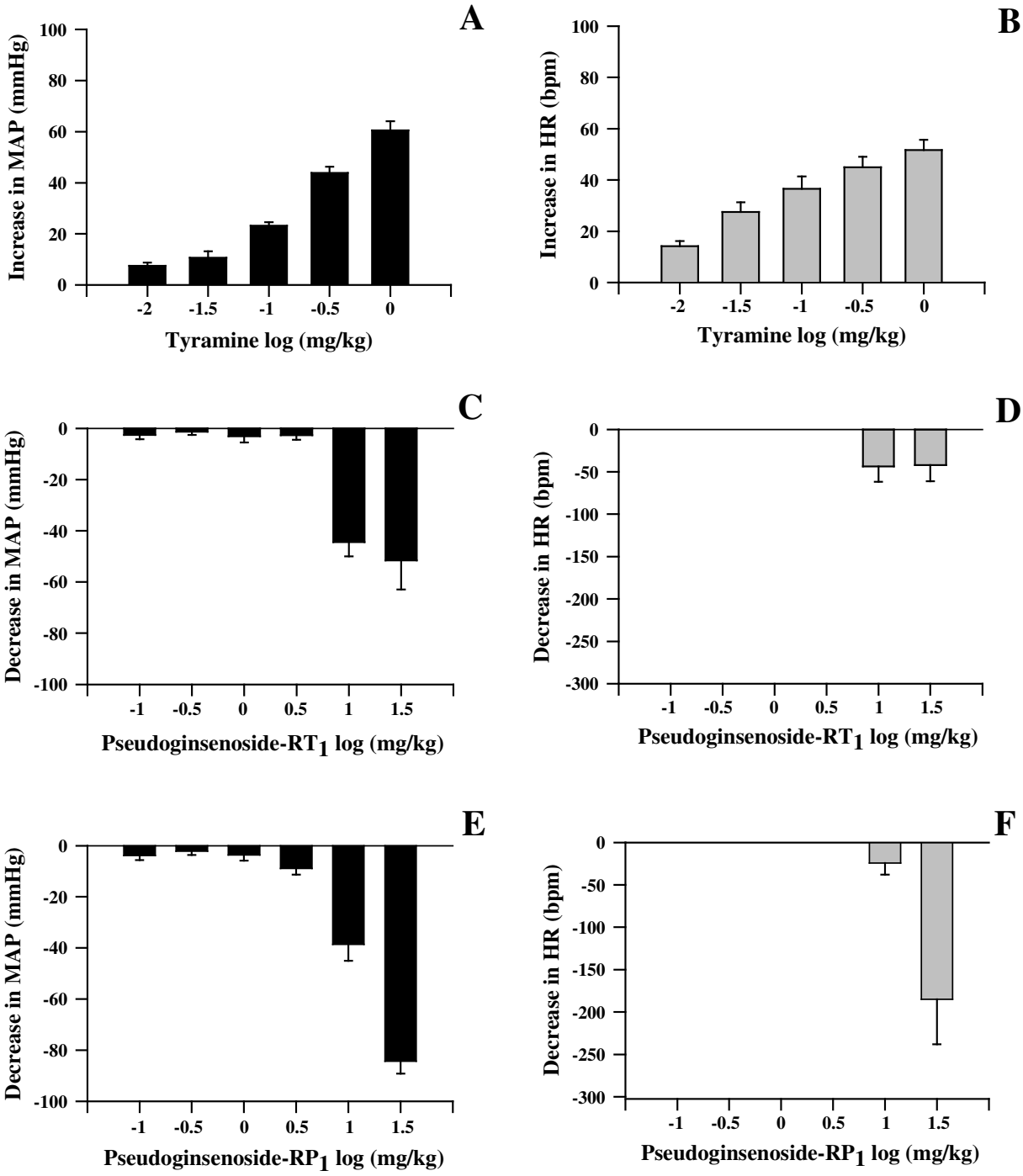

A
Increase in MAP (mmHg)
100
80
60
40
20
0
-2
-1.5
-1
-0.5
0
Tyramine log (mg/kg)
B
Increase in HR (bpm)
100
80
60
40
20
0
-2
-1.5
-1
-0.5
0
Tyramine log (mg/kg)
C
Decrease in MAP (mmHg)
0
-20
-40
-60
-80
-100
-1
-0.5
0
0.5
1
1.5
Pseudoginsenoside-RT1 log (mg/kg)
D
Decrease in HR (bpm)
0
-50
-100
-150
-200
-250
-300
-1
-0.5
0
0.5
1
1.5
Pseudoginsenoside-RT1 log (mg/kg)
E
Decrease in MAP (mmHg)
0
-20
-40
-60
-80
-100
-1
-0.5
0
0.5
1
1.5
Pseudoginsenoside-RP1 log (mg/kg)
F
Decrease in HR (bpm)
0
-50
-100
-150
-200
-250
-300
-1
-0.5
0
0.5
1
1.5
Pseudoginsenoside-RP1 log (mg/kg)

Figure 35 Effects of tyramine (A and B), pseudoginsenoside-RT_1 (C and D) or pseudoginsenoside-RP_1 (E and F) on mean arterial blood pressure (MAP, left) and heart rate (HR, right) in anesthetized rats. Each point represents mean ± S.E.M. of 6 experiments.

3.5.3 Effects of pseudoginsenosides on vasodilatation of isolated rat thoracic aortae and mesenteric arteries

Pseudoginsenoside-RT_1 (10^{-7}-10^{-2} M) had no vasodilatory effects on endothelium-intact thoracic aortae, preconstricted with $3x10^{-6}$ M phenylephrine (data not shown).

Typical recordings of the relaxant effects of pseudoginsenoside-RT_1 or pseudoginsenoside-RP_1 on the endothelium-intact mesenteric artery preconstricted with 10^{-6} M phenylephrine are shown in Figure 36.

As shown in Figure 37, pseudoginsenoside-RT_1 (10^{-7}-10^{-2} M) and pseudoginsenoside-RP_1 (10^{-5}-10^{-3} M) caused a concentration-dependent relaxation of the endothelium-intact mesenteric arteries which were preconstricted with 10^{-6} M phenylephrine. These effects persisted after removal of the vascular endothelium. Whereas pseudoginsenoside-RT_3 and -RT_5 (10^{-7}-10^{-2} M) had no relaxant effects on the mesenteric arteries which were preconstricted with phenylephrine whether the endothelium presence or not (data not shown).

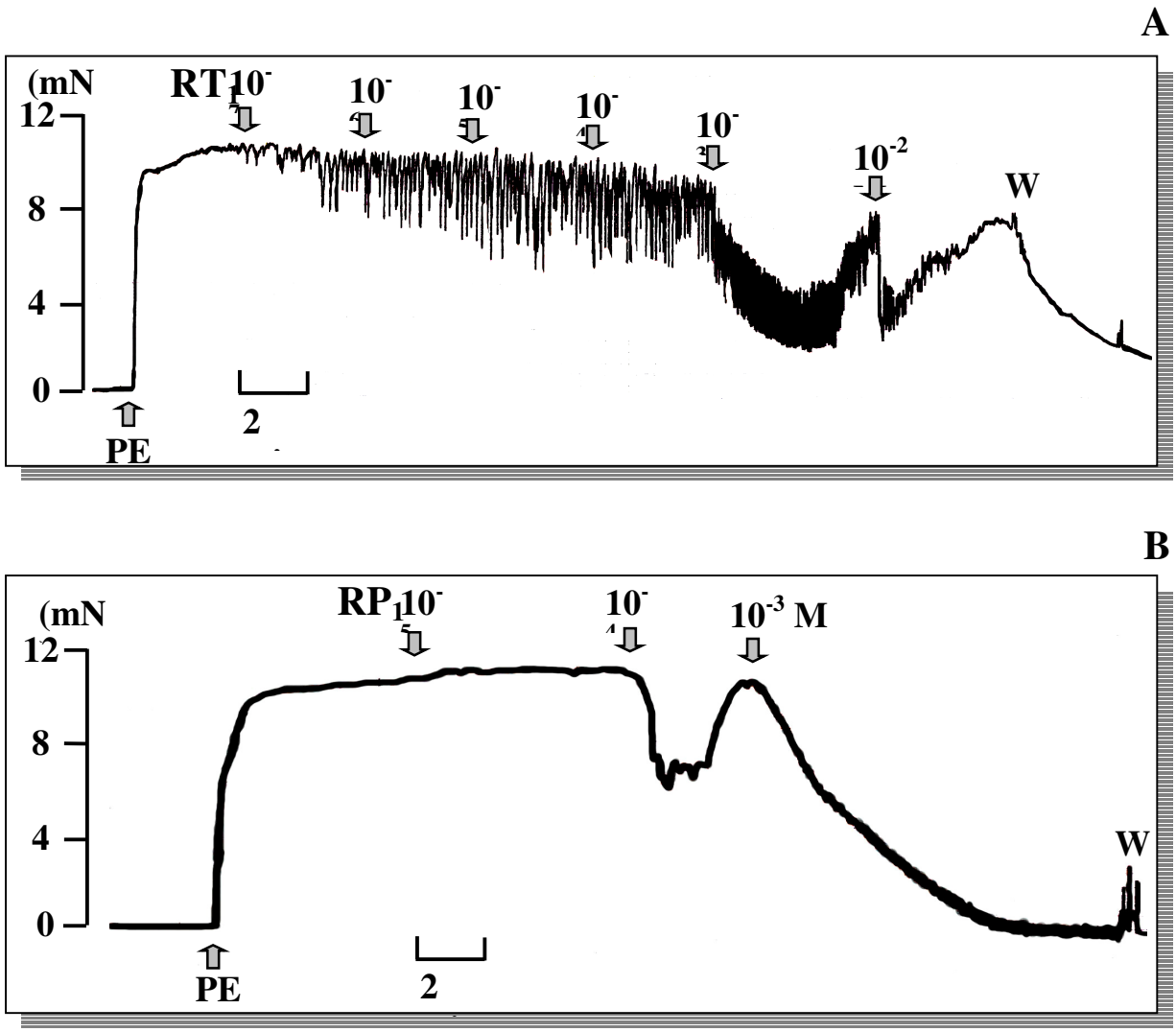


Figure 36 Typical recording showing a cumulative addition of pseudoginsenoside-RT_1 (10^{-7}-10^{-2} M) (A) and pseudoginsenoside-RP_1 (10^{-5}-10^{-3} M) (B) caused a concentration-dependent relaxation of the endothelium-intact rat mesenteric artery, preconstricted with 10^{-6} M phenylephrine. ⇩ indicates drug added in Molar (M) of the final organ bath concentration. At W, the bath solution was exchanged by washing with PSS.

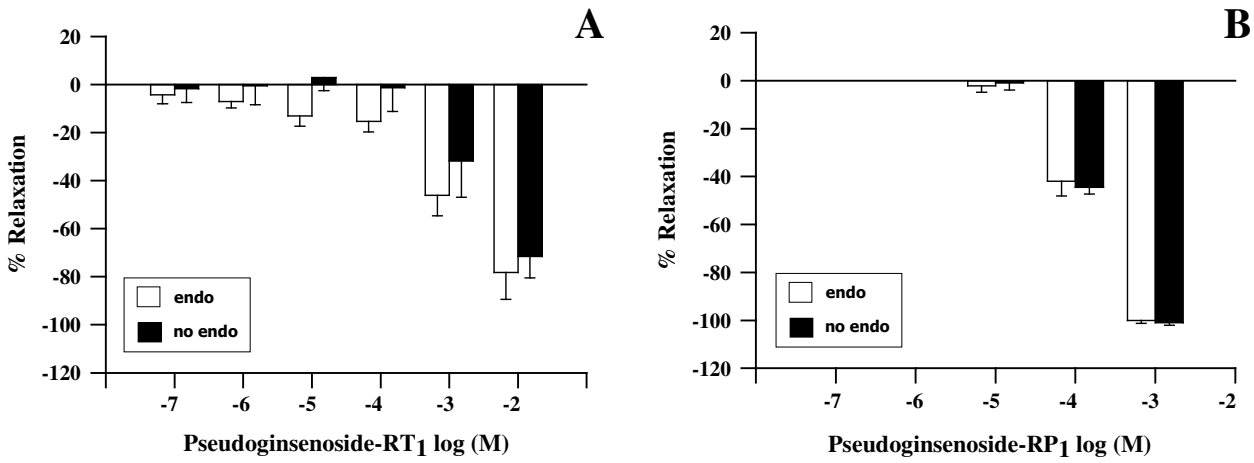


Figure 37 Effects of pseudoginsenoside-RT_1 (A) and pseudoginsenoside-RP_1 (B) on the dilator response of endothelium-intact and -denuded mesenteric arteries, preconstricted with 10^{-6} M phenylephrine. Each point represents mean ± S.E.M. of 3 experiments.

Table 1 Amounts and cardiovascular effects of compounds isolated from the *Randia siamensis* extract.

Compounds (code)	**Name of compounds**	**Total amounts (mg)**	**% Total amounts of 50-g crude ext**	**Cardiovascular effects in anesthetized rats**
Compound 1 (RS1)	Kaempferol-3-O-□-D-xylose (1-2)-□□-D-galactoside	23.6	0.047	-
Compound 2 (RS2)	Kaempferol-3-O-□-D-galactoside	27.1	0.054	-
Compound 3 (RS3)	Pseudoginsenoside-RP_1	535.5	1.071	Decreased MAP and heart rate
Compound 4 (RS4)	Pseudoginsenoside-RT_1 methyl ester	26.8	0.054	-
Compound 5 (RS5)	Tyramine	259.1	0.518	Increased MAP and heart rate
Compound 6 (RS6)	Pseudoginsenoside-RT_1	945.9	1.892	Decreased MAP and heart rate
Compound 7 (RS7)	Pseudoginsenoside-RT_5 (new)	270.6	0.541	No effect on MAP and heart rate
Compound 8 (RS8)	Pseudoginsenoside-RT_3 (new)	299.9	0.599	No effect on MAP and heart rate
Compound 9 (RS9)	5-O-[Z] caffeoylquinic acid	32.5	0.065	-
Compound 10 (RS10)	Pseudoginsenoside-RT_4 (new)	22.4	0.045	-

Compound 11 (RS11)	Pseudoginsenoside-RT_2 (new)	18.0	0.036	-

3.6 Discussion

Bioactivity-guided isolation was undertaken and 11 compounds were isolated from the crude n-butanol extract from fresh fruits of *R. siamensis*. Four new pseudoginsenoside compounds were obtained, pseudoginsenoside-RT_2, -RT_3, -RT_4, and -RT_5, together with 7 known compounds: tyramine, pseudoginsenosides-RT_1 methyl ester, pseudoginsenosides-RT_1, pseudoginsenoside-RP_1, kaempferol-3-O-β-D-xylose(1-2)-β-galactose, kaempferol-3-O-β-D-galactose, and 5-O-[Z] caffeoylquinic acid. The major components obtained from this separation are tyramine, pseudoginsenoside-RP_1, -RT_1, -RT_3 and -RT_5 that represented at 0.52, 1.07, 1.89, 0.6, and 0.54% of 50-g the *R. siamensis* extract, respectively. The other 6 components were presented approximately at 0.03-0.07 %.

As shown in the isolation section, the mixture compound tyramine (RS5) and pseudoginsenoside-RT_1 (RS6) was purified by semi-preparative HPLC using MeOH-H_2O-0.05%TFA as mobile phase (Figure 31). From this method, yielding of each compound was only small amount (Table 1) that was limited to investigate the cardiovascular mechanisms in the pharmacological study. Therefore, in order to obtain a larger amount of both tyramine and pseudoginsenoside-RT_1, further isolation was performed by MPLC (Moderate Pressure Liquid Chromatography) using a bigger column chromatography and the same mobile phase, MeOH-H_2O-0.5%TFA. However, we found that the major compound, pseudoginsenoside-RT_1, was degraded by 0.05% TFA during this separation.

Due to limited availability of the pure substances, therefore, only the major constituents were allowed to test their activities on blood pressure and heart rate in anesthetized rats. As shown in the results section, tyramine caused an increase in MAP and heart rate, whereas pseudoginsenoside-RT_1 and -RP_1, but not -RT_3 and -RT_5, caused a decrease in MAP and heart rate (Figure 35). However, the hypotensive effects of both pseudoginsenosides at the highest dose (30 mg/kg) were different. Pseudoginsenoside-RT_1 produced a prolong decrease in MAP with slightly decreased heart rate (Figure 34B), whereas pseudoginsenoside-RP_1 caused transient decrease in MAP with prolong decrease in heart rate (Figure 34C). These findings indicate that the mechanisms related to the hypotensive activity of pseudoginsenoside-RT_1 and -RP_1 is probably different. However, further studies would need to clarify this possibility.

Pseudoginsenoside-RT_1 had no relaxant activity on thoracic aortae preconstricted with phenylephrine (data not shown). However, when resistance arteries: mesenteric arteries approximate internal diameter 200-300 □m, were used, the pseudoginsenoside-RT_1 and -RP_1 caused a vasodilatation on the mesenteric arteries preconstricted with phenylephrine (Figure 37) whether the endothelium present or not. These results suggest that the hypotensive activity of the pseudoginsenosides-RT_1 and -RP_1 may be a direct action on the resistance vessel and causes vasodilatation.

3.7 Conclusion

The crude n-butanol extract from fresh fruits of *R. siamensis* extract contained 4 new pseudoginsenoside compounds: pseudoginsenosides-RT_2, -RT_3, -RT_4 and -RT_5, together with 7 known compounds: tyramine, pseudoginsenosides-RT_1 methyl ester, pseudoginsenosides-RT_1, pseudoginsenoside-RP_1, kaempferol-3-O-β-D-xylose(1-2)-□β-galactose, kaempferol-3-O-β-D-galactose, and 5-O-[Z] caffeoylquinic acid.

Tyramine is responsible for the hypertensive and positive chronotropic effects. Whereas pseudoginsenosides-RT_1 and -RP_1 are responsible for the hypotensive activity of the extract.