Research on the *in vitro* vasorelaxant constituents of Sanoshashinto and antihypertensive effects in spontaneously hypertensive rats

2019 Ph.D. Dissertation

Jianbo Wu

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Table of Contents

Abbreviation

ACE	Angiotensin-converting enzyme
ARBs	Angiotensin receptor blockers
Ba^{2+}	Barium chloride dihydrate
BB	Baicalin-berberine
BK_{Ca}	Large-conductance Ca^{2+}-activated K^{+}
Bu	*n*-butanol-
CaM	Calmodulin
CCBs	Calcium channel blockers
cGMP	Cyclic guanosine monophosphate
CHL	*Coptidis Rhizoma*
CHLM	CHL methanol extract
CHLM-W	The water fraction of CHL methanol extract
CHLM-Bu	The *n*-butanol fraction of CHL methanol extract
CHLM-EA	The ethyl acetate fraction of CHL methanol extract
CI	Cardiac index
CMCNa	Sodium carboxymethylcellulose
CO	Cardiac output
CPI-17	C-kinase potentiated protein phosphatase-1 inhibitor of 17 kD
DAD	Diode array detector
DAG	Diacylglycerol
DBP	Diastolic blood pressure
DH	*Rhei Rhizoma*
DHM	DH methanol extract
DHM-W	The water fraction of DH methanol extract
DHM-Bu	The *n*-butanol fraction of DH methanol extract
DHM-EA	The ethyl acetate fraction of DH methanol extract
DHHQM	DH and HQ methanol extract
DHHQM-W	The water fraction of DH and HQ methanol extract
DHDQM-Bu	The *n*-butanol fraction of DH and HQ methanol extract
DHHQM-EA	The ethyl acetate fraction of DH and HQ methanol extract
DHHLM	DH and HL methanol extract
DHHLM-W	The water fraction of DH and HL methanol extract
DHDLM-Bu	The *n*-butanol fraction of DH and HL methanol extract

DHHLM-EA	The ethyl acetate fraction of DH and HL methanol extract
EA	Ethyl acetate-
EC_{50}	50% effective concentration
GTP	Guanosine triphosphate
HE	Hematoxylin-eosin staining
High K^+	High concentration of potassium chloride
HPLC	High Performance Liquid Chromatography
HQ	*Scutellariae Radix*
HQM	HQ methanol extract
HQM-W	The water fraction of HQ methanol extract
HQM-Bu	The *n*-butanol fraction of HQ methanol extract
HQM-EA	The ethyl acetate fraction of HQ methanol extract
HQHLM	HQ and CHL methanol extract
HQHLM-W	The water fraction of DH and CHL methanol extract
HQDLM-Bu	The *n*-butanol fraction of DH and CHL methanol extract
HQHLM-EA	The ethyl acetate fraction of DH and CHL methanol extract
IP_3	Inositol trisphosphate
K_{ATP}	ATP-sensitive potassium channel
K_{IR}	Inward rectifier potassium channel
K_V	Voltage-gated K^+ channel
L-NAME	N^G-nitro-L-arginine methyl ester hydrochloride
LVHI	Left ventricular hypertrophy index
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MLC20	20 kDa myosin light chain
MLR	Multiple linear regression
NA	Noradrenaline
NO	Nitric oxide
NOS	Nitric oxide synthetase
PCA	Principal component analysis
PC-1	The first PC
PC-2	The second PC
PCR	Principal component regression
PCs	Principal components
PIP_2	Phosphatidylinositol (4,5)-bisphosphate

PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
Rho	Rho-associated kinase
ROCC	Receptor-operated calcium channel
ROCK	Rho-associated protein kinase
RyR	Ryanodine receptor
SBP	Systolic blood pressure
SHXXT	Sanoshashinto
SHXXTM	SHXXT methanol extract
SHXXTM-W	The water fraction of SHXXT methanol extract
SHXXTM-Bu	The *n*-butanol fraction of SHXXT methanol extract
SHXXTM-EA	The ethyl acetate fraction of SHXXT methanol extract
SHXXTW	The water extract of SHXXT
SHXXTM-PHPLC-BB	The baicalin and berberine part of SHXXTM
SHXXTM-PHPLC-except BB	SHXXTM except baicalin and berberine part
SHRs	Spontaneously hypertensive rats
sGC	Soluble guanylyl cyclase
SR	Sarcoplasmic reticulum
SVR	Systemic vascular resistance
TEA	Tetraethylammonium chloride
W	Water-
VDCC	Voltage-dependent calcium channel
1-ANOVA	One-way analysis of variance
4-AP	4-Aminopyridine

Abstract

Antihypertensive drugs, usually in the market, are synthetic drugs which reduce the blood pressure rapidly. With the long-time using of synthetic drugs by patients, the side effects have gradually become manifest. For this reason, researchers have sought to find drugs having low side effects and shifted their focus onto the traditional and folk medicines, which are expected to produce good vasorelaxant and antihypertensive effects. But these medicines are complicated drugs containing a lot of components and hard to be elucidated. Sanoshashinto, also called SanHuangXieXinTang (SHXXT) in China, originated in the Essential Prescriptions of the Golden Cabinet (Jin Kui Yao Lue). It is composed of three materials: *Rhei Rhizoma* (DaHuang in Chinese medicine, DH), *Scutellariae Radix* (HuangQin in Chinese medicine, HQ), and *Coptidis Rhizoma* (Chinese HuangLian in Chinese medicine, CHL), and has been used to lower body temperature and dissipate body dampness since ancient times. Recently, it was found that SHXXT could relax vascular contractions *in vitro* and lower blood pressure in patients, but detailed research of SHXXT are few. Therefore, in this study, connecting HPLC and principal component analysis (PCA), *in vitro* and *in vivo* experiments, a detailed research about vasorelaxant and antihypertensive effects of SHXXT were proceeded.

Chapter I: Vasorelaxant effects of extracts and fractions on the *in vitro* experiments

SHXXT, each material (DH, HQ, CHL), and different combinations (DH and HQ, DH and CHL, HQ and CHL) were extracted with methanol and fractionated into water-, *n*-butanol-, and ethyl acetate-fractions. Then effects of all extracts and fractions (total 28 samples) on high-concentration of KCl (High K^+)- or noradrenaline (NA)-induced contractions of isolated rat aortic rings or helical strips were examined. The results showed that, in the endothelium-denuded strips, SHXXT methanol extract (SHXXTM) relaxed the NA-induced vascular contraction with EC_{50} of 16.2 ± 1.1 μg/mL, and relaxed the High K^+-induced vascular contraction with EC_{50} of 65.1 ± 5.5 μg/mL. In the endothelium-intact rings, SHXXTM relaxed the NA-induced vascular contraction with EC_{50} of 10.5 ± 0.1 μg/mL.

Chapter II: The results of HPLC analysis and PCA

Total 39 peaks were detected in SHXXTM. Among of them, 11 compounds were identified compared with the reference standards, and the amounts of baicalin, berberine, wogonoside, coptisine, baicalein, and palmatine were higher than the others. The other extracts and fractions were also analyzed by HPLC.

All HPLC analysis and *in vitro* experimental data were analyzed by PCA software. In the PCA calculation, a data matrix which contained 28 objects and 39 variables was used to examine the Loading factors which can reveal the important variables for the main variation in the data. The results showed that the first two principal components (PCs) could summarize 77% of the HPLC information, and on

the PC-1 axis, peak No.33 (berberine) and No.32 (palmatine) had the most positive Loading-1 factor, while peak No.18 (baicalin), No.24 (wogonoside) and No.31 (baicalein) had the negative Loading-1 factor. Peak No.18, No.24, and No.33 were comparatively larger in the Loading-2 factor than others. Connected all HPLC analysis and EC_{50} data, principal component regression analysis was used to calculate whether the results were reliable or not. The results showed that the values of predicated EC_{50} and experimentally observed EC_{50} were almost same, and regression coefficients were larger than 0.99. From these results, baicalin, berberine, palmatine, baicalein, and wogonoside were thought to contribute significantly to the pharmacological activity. The contents of baicalin and berberine were relatively higher than the others, and also the results in the *in vitro* experiments showed that the baicalin-berberine (BB) combination had almost the same EC_{50} (15.3 ± 0.7 μg/mL) compared with SHXXTM. Therefore, the BB combination were considered as the main antivascular contraction constituents and could replace SHXXTM *in vitro* experiments.

Chapter III: ***In vitro* mechanism research and *in vivo* study**

To clarify which channels or pathways are involved in the vasorelaxant effects, I conducted preliminary *in vitro* experiments using the BB combination (the concentrations of BB were equivalent to SHXXTM) and several activators or inhibitors. The results showed that in the case of pretreatment with N^G-nitro-L-arginine methyl ester hydrochloride, an inhibitor of nitric oxide synthetase (NOS), the vasorelaxant effects were obviously reduced; pretreatment with diazoxide, a K_{ATP} channel activator, the vasorelaxant effects were slightly increased; pretreatment with rottlerin, a large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel activator, and calphostin C, a protein kinase C inhibitor, the vasorelaxant effects were increased, especially calphostin C. These results showed that the BB combination produced vasorelaxant effects by activating the NO/cGMP pathway in the endothelium-intact rings, and the BK_{Ca} channel, K_{ATP} channel, and the DAG/PKC/CPI-17 pathway might be involved.

With regard to *in vivo* study, from the fourth week onward, SHXXTM (200–800 mg/kg/d, *p.o.*) and the BB combination (32 + 26 mg/kg/d, *p.o.*) significantly reduced the increase in the rate of systolic blood pressure and left ventricular hypertrophy in spontaneously hypertensive rats. The results indicated that the SHXXTM and the BB combination exhibited significant antihypertensive effects *in vivo*.

Conclusion:

In this study, I provided the first piece of evidence that baicalin and berberine are the main effective constituents in SHXXT. Furthermore, several mechanisms of action by the BB combination were revealed using several agonists and inhibitors. All of the results indicated that SHXXTM exhibited significant antihypertensive effects, and the BB combination could replace SHXXT for use as an antihypertensive drug in the future.

Introduction

With the improvement of the standard of living worldwide, more and more people are being diagnosed with hypertension, and hypertension has become a global public health issue. It is estimated that more than 1 billion adults have hypertension and the number is increasing yearly [1] (Figure 1).

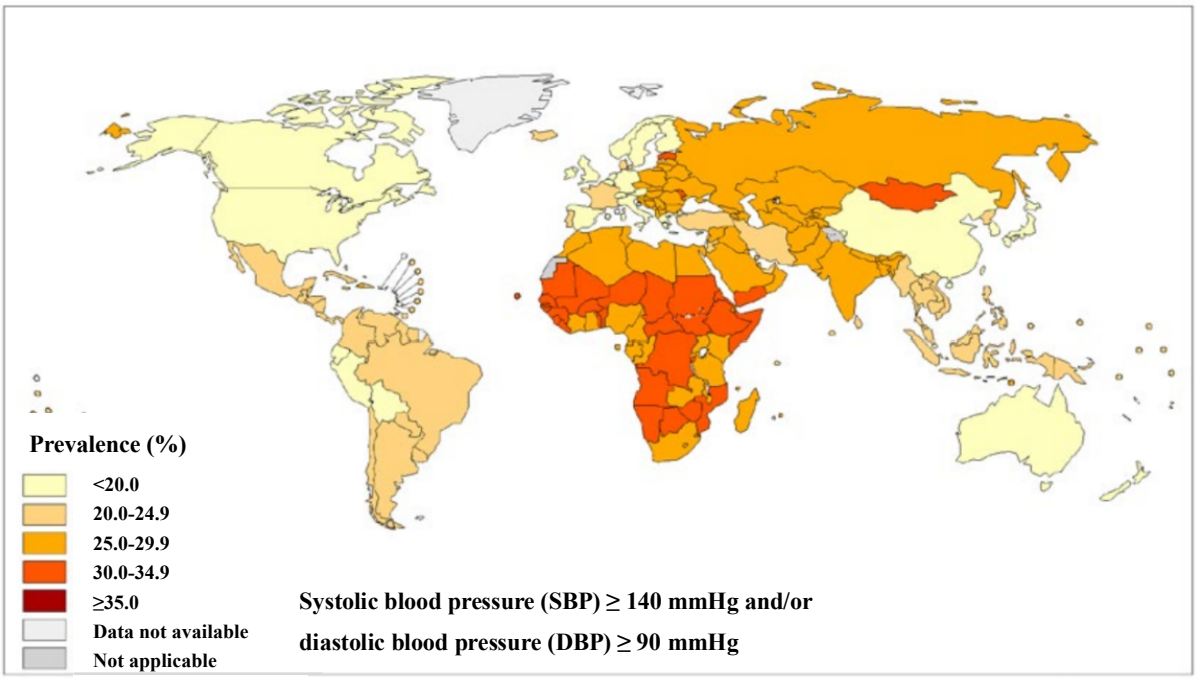


Figure 1 Prevalence of hypertension in all of the world (WHO data, 2015) [2]

Hypertension is defined as systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg (Table 1) [3], and is calculated by multiplying systemic vascular resistance (SVR) and cardiac output (CO) (BP = SVR × CO) [4]. Therefore, an increase in either SVR or CO would result in hypertension (Figure 2).

Table 1 Classification of blood pressure

Category	Systolic (mmHg)		Diastolic (mmHg)
Optimal	<120	and	<80
Normal	120~129	and/or	80~84
High normal	130~139	and/or	85~89
Grade 1 hypertension	140~159	and/or	90~99
Grade 2 hypertension	160~179	and/or	100~109
Grade 3 hypertension	≧180	and/or	≧110

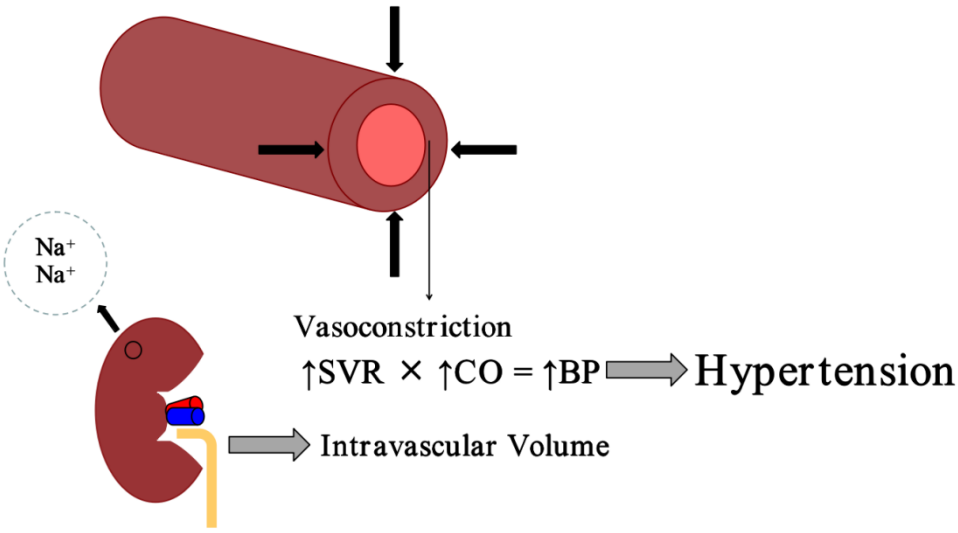


Figure 2 The main changes causing hypertension

Hypertension is considered as a complex disease that may be caused by many factors, including genetic inheritance, high sodium intake, alcohol, smoking, and so on [5-7]. The hypertensive patients also need to take the drugs to control the blood pressure in all of the life. Besides this, hypertension is also the most important contributor to cardiovascular disease, including ischemic stroke, myocardial infarction, heart failure, and peripheral artery diseases [8, 9]. Today, hypertension and cardiovascular risk are usually assessed together to help physicians make the correct diagnosis and prescribe the correct drugs. Although hypertension is very complicated and refractory, but many drugs were developed to treat hypertension, such as angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), beta-blockers, calcium channel blockers (CCBs) and diuretics, and these drugs are used singly or in combination [10, 11]. Recently, with the long-time using of drugs by patients, the side effects of hypertension drugs have gradually become manifest. For example, ACE inhibitors and ARBs cause cough and angioedema and functional renal insufficiency, beta-blockers usually cause insomnia, hallucinations, and depression, and CCBs usually cause ankle edema, headache, flushing, and tachycardia, diuretics usually cause hyponatremia and hypokalemia (Table 2) [10, 12]. Although the side effects are not very serious, researchers have sought to find drugs having low side effects and shifted their focus onto traditional medicines and folk medicines, which are expected to produce good vasorelaxant and antihypertensive effects [13-15].

Table 2 Classification of antihypertensive drugs

Classification	**Drugs**	**Side effects**
Angiotensin-converting enzyme (ACE) inhibitors	Benazepril Captopril…	cough, angioedemas, functional renal insufficiency
Angiotensin receptor blockers (ARBs)	Candesartan Eprosartan…	cough, angioedemas, functional renal insufficiency
Beta-blockers	Acebutolol Atenolol…	insomnia, hallucinations, depression
Calcium channel blockers (CCBs)	Diltiazem Verapamil…	ankle edema, headache, flushing, tachycardia
Diuretics	Furosemide Bumetanide…	hyponatremia, hypokalemia

Sanoshashinto, which was called SanHuangXieXinTang (SHXXT, 三黄瀉心湯) in China, originated in the Essential Prescriptions of the Golden Cabinet (Jin Kui Yao Lue, 金匱要略). It is composed of three materials: *Rhei Rhizoma* (DaHuang in Chinese medicine, DH, 大黄), *Scutellariae Radix* (HuangQin in Chinese medicine, HQ, 黄芩), and *Coptidis Rhizoma* (Chinese HuangLian in Chinese medicine, CHL, 黄連), and has been used to lower body temperature and dissipate body dampness since ancient times. Recently, it was found that SHXXT could relax vascular contractions *in vitro* and lower blood pressure in patients [16-19]. Chen and Hsieh reported that in the clinical study, when the patients were orally taken SHXXT extract 500 or 750 mg/day, the SBP and DBP would reduce obviously (from 154/102 to 138/89 in 1 month, and 158/105 to 136/87 in 2 years) [18, 19]. DH contains rhein, emodin, and sennoside A, and acts by lowering serum cholesterol, improving diabetic nephropathy, and exerting an anti-inflammatory effect [20, 21]. HQ contains baicalin, baicalein, wogonin, and other flavonoids, and produces antihypertensive, anti-inflammatory, and antioxidant effects. Baicalin, the main constituent of HQ, induces rat mesenteric arterial relaxation [22, 23]. CHL contains berberine, palmatine, and other alkaloids, and exerts anti-inflammatory, antihypertensive, antihyperglycemic, antiarrhythmic, and antidepressant effects [21, 24]. Berberine, the main constituent of CHL, shows good vasorelaxant effects in the mesenteric artery of rat [25, 26]. Although SHXXT, HQ, and CHL exhibit good antihypertensive and vasorelaxant effects individually, detailed investigations of the antihypertensive and vasorelaxant effects of their components, and the possible mechanisms of effects are few. Therefore, in this study, a mixture of the three materials and combinations of SHXXT were extracted with methanol and fractionated, and all the extracts and the fractions were tested on *in vitro* antivascular contraction experiments and also analyzed by HPLC. Then, all *in vitro* experiments and HPLC analysis data were analyzed by PCA software to speculate the constituents which were important and responsible for vasorelaxation. Furthermore, the extracts and the mixture of the main important constituents were administered to

spontaneously hypertensive rats (SHRs) to check whether blood pressure was modulated. Meanwhile, the hearts of rats were removed and determined the left ventricular hypertrophy index (LVHI) and the cardiac index (CI). Also a microscopic examination of the aorta was performed to verify whether SHXXT had comprehensive effects on SHRs. Besides these, the possible underlying mechanisms of action *in vitro* were also discussed.

Chapter I: Vasorelaxant effects of SHXXT extracts and fractions on the *in vitro* experiments

1. Materials and methods

1.1 Materials

The three crude drugs, DH, HQ, and CHL, used in this study, were purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan). The lot numbers of DH, HQ, and CHL used in this study are 007016001, 001116002, and 001317001, respectively.

1.2 Chemicals

Methanol, acetonitrile, and dimethyl sulfoxide (DMSO) for sample preparation are of HPLC grade. All other chemicals and reagents are of analytical reagent grade.

1.3 Animals

All procedures and protocols (No: PCOG-17-008) were approved by the Animal Care and Use Committee of Kyoto Pharmaceutical University. Male Sprague-Dawley rats (200–300 g, 7–8 weeks) were housed under constant temperature and illumination conditions. The rats were allowed access to food and water ad libitum. All rats were purchased from Japan SLC, Inc. (Shizuoka, Japan).

1.4 Methods

1.4.1 The extraction of SHXXT

A blended mixture of DH, HQ, and CHL in 1:1:1 ratio [27] was refluxed with methanol (10 times volume) for 1.5 h, and this procedure was repeated three times. The product collected by refluxing was filtered. The filtrate was concentrated under reduced pressure at 40 ℃ to obtain the solid extract.

Meanwhile, for PCA, each material and combinations of two materials (DH and HQ, DHHQ; DH and CHL, DHHL; HQ and CHL, HQHL) were also extracted by the same method and fractionated into water- (W), *n*-butanol- (Bu), and ethyl acetate- (EA) fractions by the separating funnel. All samples were stored at 4 ℃ for the next experiments.

1.4.2 Tissue preparation and flow chart of *in vitro* antivascular experiments

Rat thoracic aorta were carefully removed and cut into 2–3 mm long rings with endothelium (hereinafter "endothelium-intact rings") or helical strips without endothelium (hereinafter "endothelium-denuded strips"; *ca*. 2 mm wide × 15 mm long). The ring preparations were set on stainless steel wires and suspended in a 5 mL bath, and the helical strips were placed on stainless steel

wires and suspended in individual 6 mL organ baths. All the baths were filled with Krebs solution with the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, and D-glucose 11. The solution was maintained at 37 °C and aerated with 95% O_2 plus 5% CO_2. Contractions of rings were measured with WinDaq Data Acquisition software (Ohio, USA) and those of helical strips were measured isometrically with a force-displacement transducer (ML T0201/D, ADInstruments Pty Ltd., New South Wales, Australia) and recorded using a software, Chart v3.6.8 for PowerLab/MacLab (ADInstruments). An one-hour equilibration period was allowed before initiation of experiments. After equilibration, 2 M High K^+ (0.18 mL, final concentration 60 mM) and 10^{-3} M NA (5 or 6 μL, final concentration 10^{-6} M) were added to the bath separately (Figures 3 and 4). The tissues were washed three times and re-equilibrated after the contractions reached a maximum. This procedure was repeated, the second contraction was obtained for the next study [28].

All SHXXT samples were dissolved in the concentration of 100 mg/mL by DMSO for *in vitro* antivascular contraction experiments. After the preparation, the samples were diluted into 30, 10, 3, 1 mg/mL, and final medium concentration of samples in medium were 100 μg/mL. The specific experiments process is shown in Figure 4.

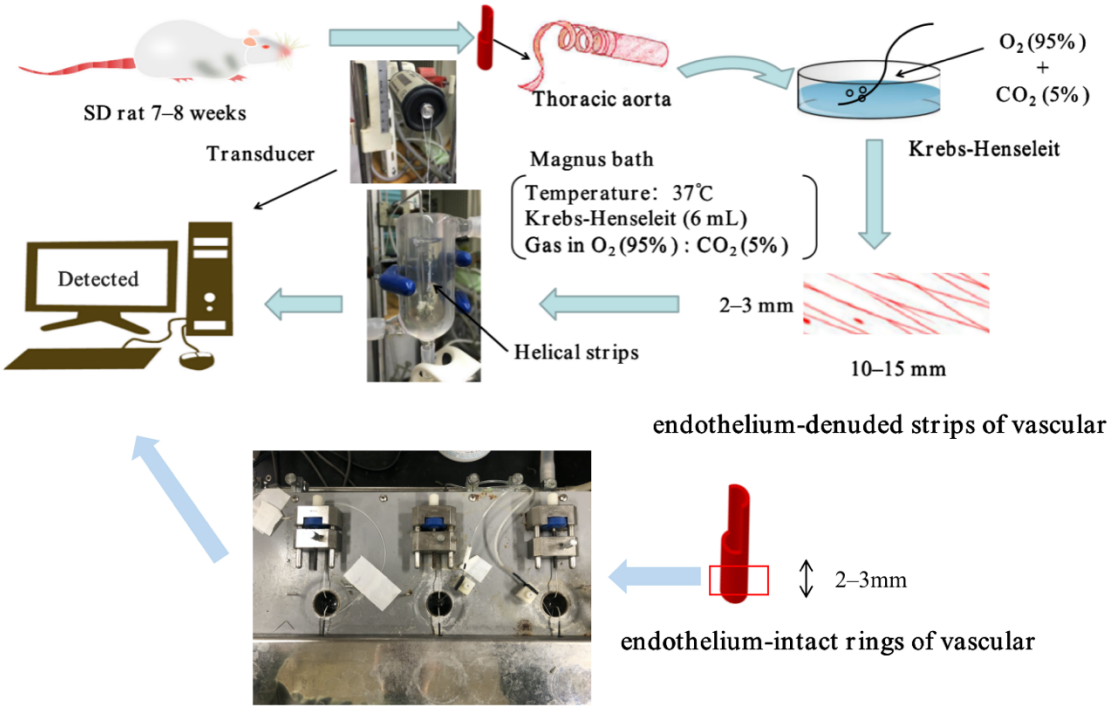


Figure 3 The process of *in vitro* antivascular experiments

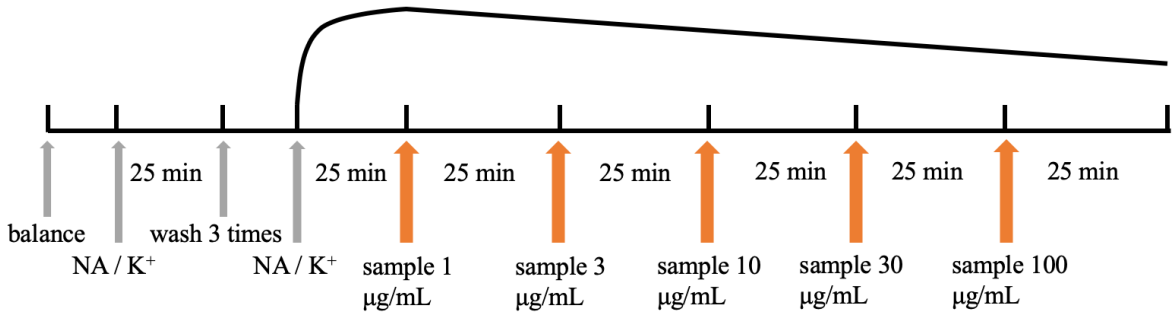


Figure 4 The specific process of *in vitro* experiments

1.5 Statistical analysis

Data were expressed as means ± S.E.M. Significant differences between groups were assessed by one-way analysis of variance (1-ANOVA) followed by Dunnett's method. A P-value less than 0.05 was considered significant.

2. Results

2.1 Results of SHXXT extracts

The yields of each material and different combinations are shown in Table 3.

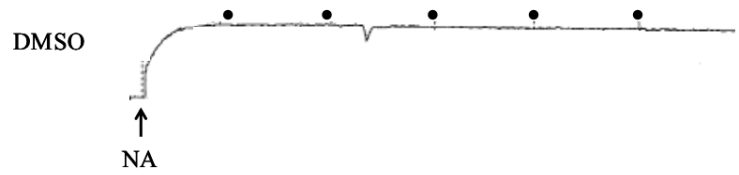
Table 3 The yields of the methanolic extracts

Extracts name	Yield (%)
SHXXT（大黄+黄芩+黄連）	24.8
DH（大黄）	35.6
HQ（黄芩）	28.3
CHL（黄連）	16.9
DH + HQ（大黄+黄芩）	31.7
DH + CHL（大黄+黄連）	22.9
HQ + CHL（黄芩+黄連）	25.8

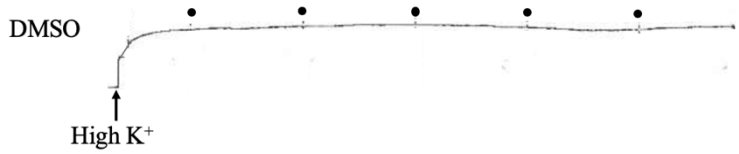
2.2 Results of SHXXT in High K^+- and NA-induced vascular contractions in the endothelium-denuded strips

In isolated aorta, the maximal tension obtained by 60 mM High K^+ and 10^{-6} M NA was considered to indicate 100% contraction (n = 4–6) relative to basal tension. In the endothelium-denuded strips, SHXXTM relaxed the NA-induced vascular contraction with an half maximal effective concentration (EC_{50}) of 16.2 ± 1.1 μg/mL, and relaxed the High K^+-induced vascular contraction with an EC_{50} of 65.1 ± 5.5 μg/mL (Figures 5 and 6).

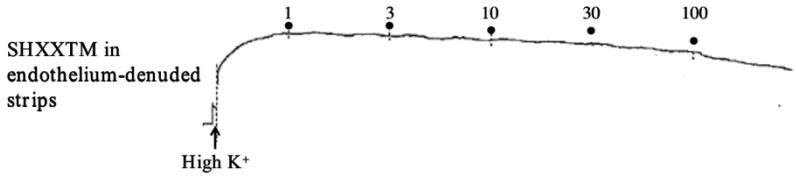
a:


DMSO
NA

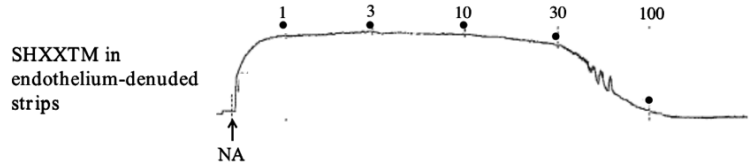
b:


DMSO
High K+

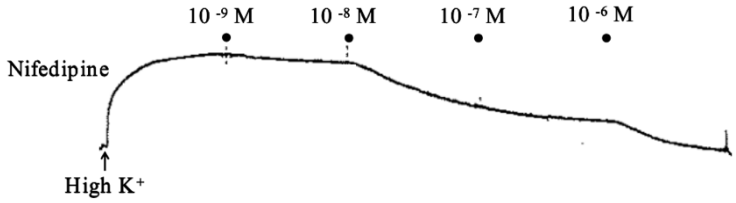
c:


1
3
10
30
100
SHXXTM in endothelium-denuded strips
High K+

d:


1
3
10
30
100
SHXXTM in endothelium-denuded strips
NA

e:


10 -9 M
10 -8 M
10 -7 M
10 -6 M
Nifedipine
High K+

f:

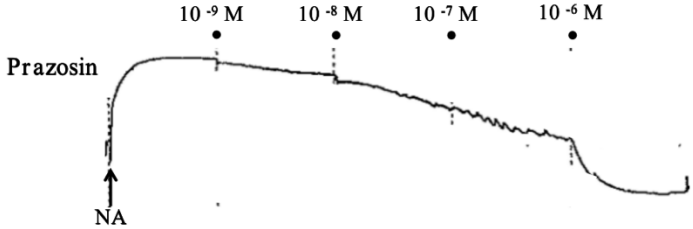

10 -9 M
10 -8 M
10 -7 M
10 -6 M
Prazosin
NA

Figure 5

a: DMSO on NA-induced contraction in endothelium-denuded strips

b: DMSO on High K^+-induced contraction in endothelium-denuded strips

c: SHXXTM on High K^+-induced contraction in endothelium-denuded strips

d: SHXXTM on NA-induced contraction in endothelium-denuded strips

e: Positive control on High K^+-induced contraction in endothelium-denuded strips

f: Positive control on NA-induced contraction in endothelium-denuded strips

[*J. Nat. Med.* (2020), Fig.3, https://doi.org/10.1007/s11418-019-01382-9]

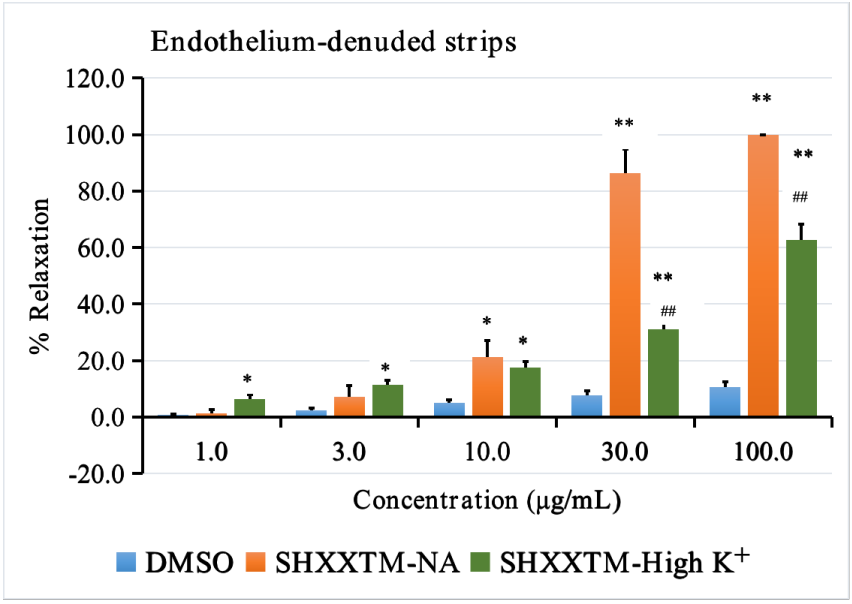


Figure 6 Vasorelaxant effects of SHXXTM on NA- and High K^+-induced contractions in the endothelium-denuded strips

Each bar graph represents the mean with S.E.M. (n = 4–6), *p<0.05 and **p<0.01 vs. DMSO control group, and $^{\#\#}p$< 0.01 vs. SHXXTM-NA group.

[*J. Nat. Med.* (2020), Fig.3, https://doi.org/10.1007/s11418-019-01382-9]

2.3 Results of SHXXT on NA-induced vascular contractions in the endothelium-intact rings

In the endothelium-intact rings, SHXXTM relaxed the NA-induced vascular contraction with an EC_{50} of 10.5 ± 0.1 μg/mL (Figures 7 and 8).

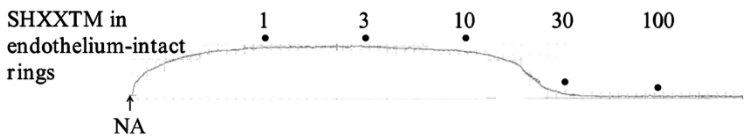


Figure 7 Graphs of SHXXTM on the *in vitro* antivascular contractions experiments in endothelium-intact rings

[*J. Nat. Med.* (2020), Fig.3, https://doi.org/10.1007/s11418-019-01382-9]

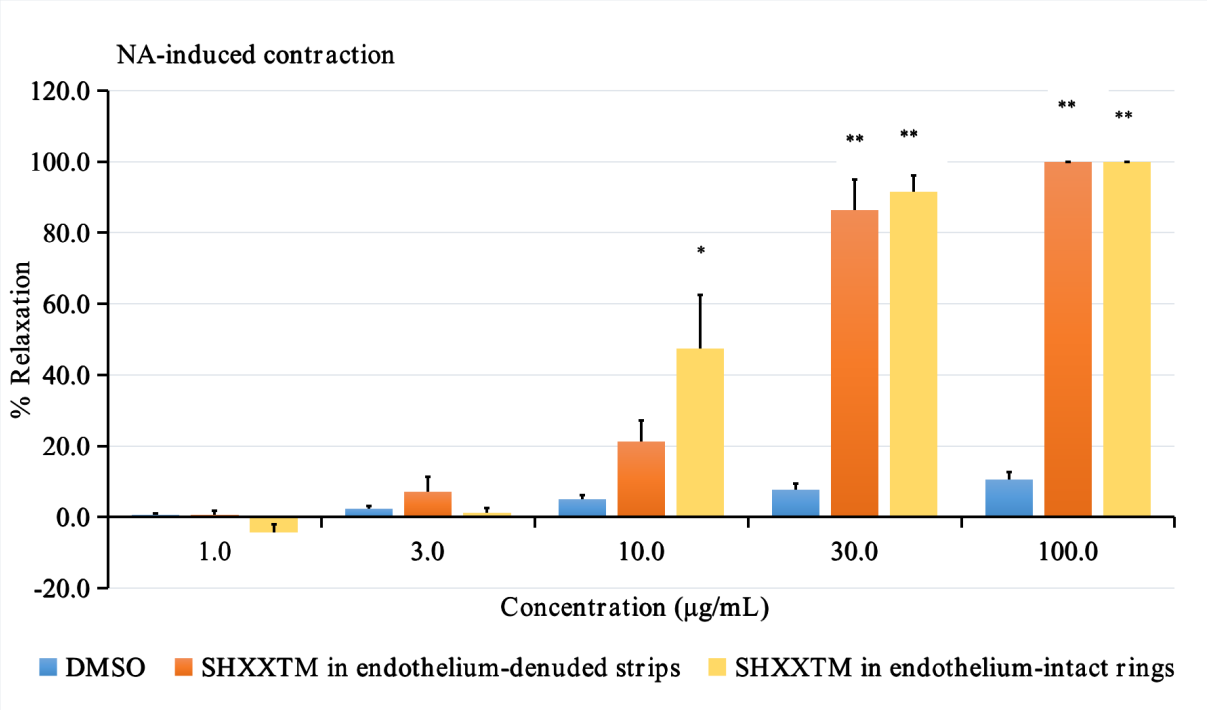


Figure 8 Vasorelaxant effects of SHXXTM on NA-induced contractions in endothelium-denuded strips and endothelium-intact rings

Each bar graph represents the mean with S.E.M. ($n = 4–6$), $*p < 0.05$ and $**p < 0.01$ vs. DMSO control group.

[*J. Nat. Med.* (2020), Fig.3, https://doi.org/10.1007/s11418-019-01382-9]

2.4 Results of all SHXXT samples on High K^+- and NA-induced vascular contractions in endothelium-denuded strips

All samples and different fractions were also tested on the High K^+- or NA-induced contractions in the isolated rat endothelium-denuded strips. The results are shown in Table. 4. Because almost all the samples and fractions showed weak effects on High K^+-induced contractions, so the data are not shown here. The vasorelaxant effects graphs of all samples and fractions on NA-induced contractions are shown in Figure S1 (Figure S1 is shown in supplementary materials).

Table 4 The vasorelaxant effects of all samples on High K^+- or NA-induced contractions in the endothelium-denuded strips

Sample	Con. (μg/mL)	NA (relaxation %) 1	3	10	30	100	EC_{50} (μg/mL)*
SHXXT	M	0.6	7.1	21.2	86.3	100.0	16.2±1.1
	W	-1.0	5.6	29.0	61.7	97.5	20.0±0.7
	Bu	-1.5	10.5	73.7	90.9	100.0	6.7±0.3
	EA	1.5	9.6	20.7	48.4	81.2	32.2±1.3
DH	M	0.0	3.5	11.0	22.7	49.5	>100
	W	3.0	8.1	10.2	14.6	28.8	>100
	Bu	2.9	5.4	13.5	21.4	41.7	>100
	EA	1.6	11.6	34.6	90.0	100.0	12.8±1.4
HQ	M	0.2	3.1	31.3	100.0	100.0	15.8±1.5
	W	0.8	3.5	36.3	100.0	100.0	15.3±1.8
	Bu	0.9	8.4	95.0	100.0	100.0	5.2±0.0
	EA	-3.2	-0.2	10.9	65.2	100.0	23.3±0.8
CHL	M	4.5	16.8	40.5	94.0	100.0	10.8±1.4
	W	4.2	21.5	53.8	92.1	100.0	8.3±0.7
	Bu	2.6	11.3	42.0	93.9	100.0	11.0±0.8
	EA	0.4	4.0	10.6	15.7	50.9	>100
DH + HQ	M	7.7	4.8	9.6	44.0	90.7	33.8±10.0
	W	7.7	9.8	13.0	62.3	95.0	24.5±0.5
	Bu	12.5	12.0	26.8	76.5	98.3	17.0±0.3
	EA	3.4	10.9	17.1	53.8	93.8	28.8±2.8
DH + CHL	M	1.5	16.4	34.4	79.2	100.0	13.9±2.3
	W	7.1	16.7	37.7	69.2	96.1	15.5±0.4
	Bu	6.6	18.5	36.3	75.8	100.0	14.1±1.5
	EA	1.8	8.4	30.4	70.6	100.0	17.4±0.3
HQ + CHL	M	0.1	11.2	72.3	100.0	100.0	6.9±0.0
	W	3.8	38.5	98.6	100.0	100.0	5.0±1.8
	Bu	-2.2	2.1	21.0	87.0	100.0	15.9±0.9
	EA	-4.2	-3.7	10.9	93.3	100.0	15.9±0.1

* Each value represents the mean with S.E.M. (n = 4–6).

3. Discussion

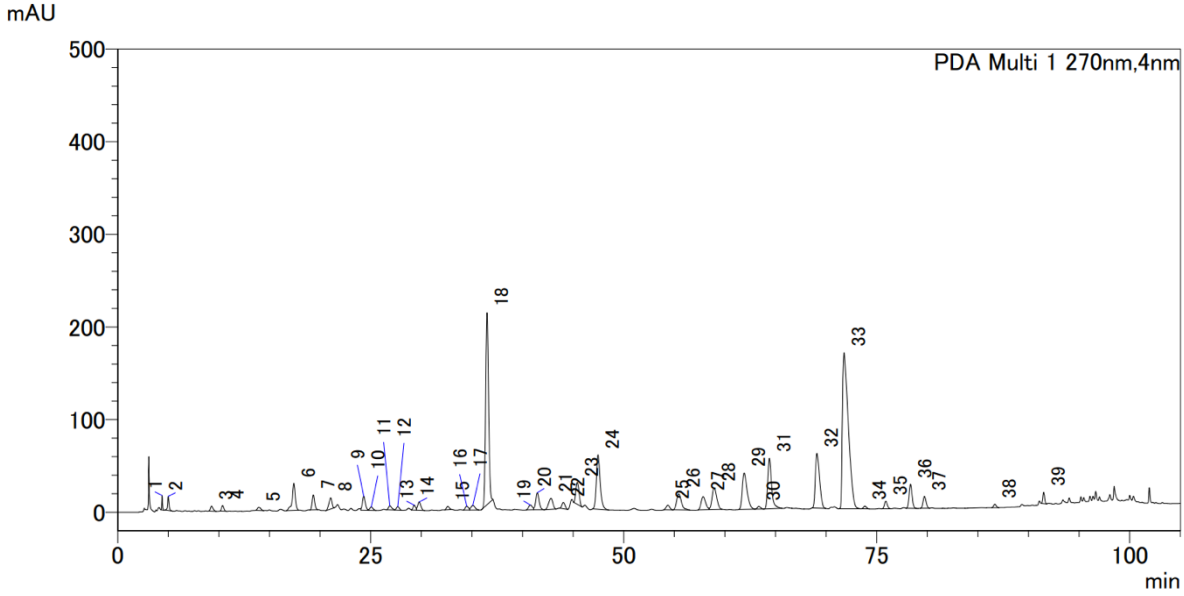
SHXXT, which is a classical prescription and very famous both in China and Japan. It was recorded that this prescription was suitable for Yo pattern (陽証) and excess pattern (実証), improving the habitus, reliefing dizziness, facial redness, nervious anxiety and constipation, and also it could ameliorate the symptoms caused by hypertension, such as dizziness, periarthritis of shoulder, tinnitus, insomnia, anxiety and so on. Besides these, it could be used in hemorrhage, for example, nose bleed and hemorrhoid bleed. In addition, Sanogan (三黄丸) and Sanousan (三黄散) composed of the same three materials have been clinically used for the same symptoms in Japan [27, 29].

Usually, in clinical, water is used to extract the samples. Here, in this study, SHXXT was also extracted by water which aimed to compared with SHXXTM, HPLC profiles and EC_{50} values of *in vitro* experiments of the SHXXTM and the water extract of SHXXT (SHXXTW) were also carried on. From the results, it was found that the number of peaks and their amounts in SHXXTW were decreased except for baicalin. This indicated that all the peaks in SHXXTW was contained in SHXXTM (Figure 9 and Table 5), but the profile of SHXXTM was observed more peaks than that of SHXXTW, because methanol could extract more liposoluble constituents than water. Meanwhile, the chromatogram showed the content of berberine in SHXXTW was decreased, because the lots of tannin in DH could combine with berberine and produce it water insoluble complex [30]. In the *in vitro* experiments, the EC_{50} values of SHXXTM (EC_{50} = 16.2 μg/mL) and SHXXTW (EC_{50} = 11.6 μg/mL) were not so different (Figure 10). Besides these reasons, various data were needed for the PCA to observe the relationship between the peaks. We therefore selected methanol as the extraction solvent. But in future, we would like to continue to do research about the SHXXTW to compare with SHXXTM.

From the results, it was found that on High K^+- and NA -induced vascular contractions in the endothelium-denuded strips, SHXXTM got good vasorelaxant effects on the NA-induced vascular contractions, while on High K^+-induced vascular contractions, the vasorelaxant effects were relatively weaker. These results indicated that agonist receptor pathway in vascular smooth muscle was might be mainly involved in the vasorelaxant effects, but voltage-dependent calcium channel was slightly involved in the vasorelaxant effects. When in the endothelium-intact rings, SHXXTM got good vasorelaxant effects than in the endothelium-denuded strips. It indicated that the endothelium was important for the vasorelaxant effects. These results were also in accordance with the previous study [17].

In all the samples and fractions, the *n*-butanol fraction of SHXXT methanol extract (SHXXTM-Bu), the *n*-butanol fraction of HQ methanol extract (HQM-Bu), the water fraction of CHL methanol extract (CHLM-W), the methanol extract of HQ and CHL(HQHLM), the water fraction of HQ and CHL methanol extract (HQHLM-W), also showed good vasorelaxant effects on *in vitro* antivascular contraction experiments. These indicated that HQ and CHL might have an important contribution to the vasorelaxant effects, while the DH might have a weak contribution.

a:



b:

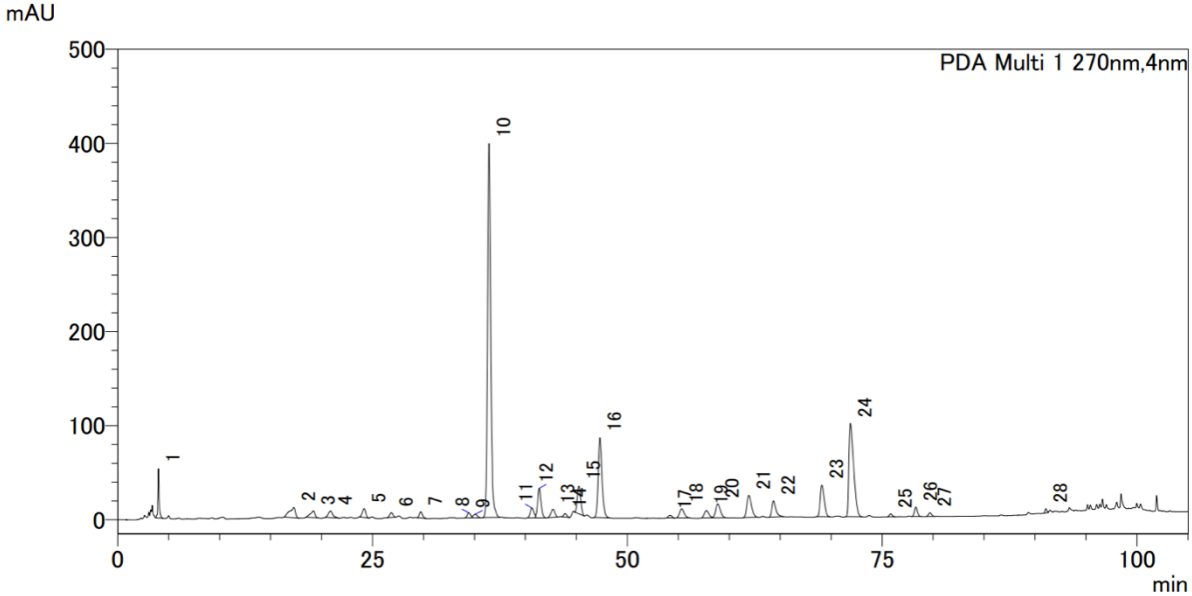


Figure 9

a: HPLC chromatogram of the methanol extract of SHXXT (SHXXTM)

b: HPLC chromatogram of the water extract of SHXXT (SHXXTW)

Table 5 The peak corresponding relation between SHXXTM and SHXXTW

Peak No and name \ Sample name	SHXXTM	SHXXTW
	1	
	2	1
	3	
	4	
	5	
	6	2
	7	3
	8	4
	9	5
	10	
	11	6
	12	
	13	
sennoside A	14	7
	15	
	16	8
	17	9
baicalin	18	10
	19	11
	20	12
	21	13
	22	14
	23	15
wogonoside	24	16
	25	17
	26	18
	27	19
	28	20
coptisine	29	21
	30	
baicalein	31	22
palmatine	32	23
berberine	33	24
rhein	34	25
	35	
	36	26
wogonin	37	27
emodin	38	
chrysophanic acid	39	28

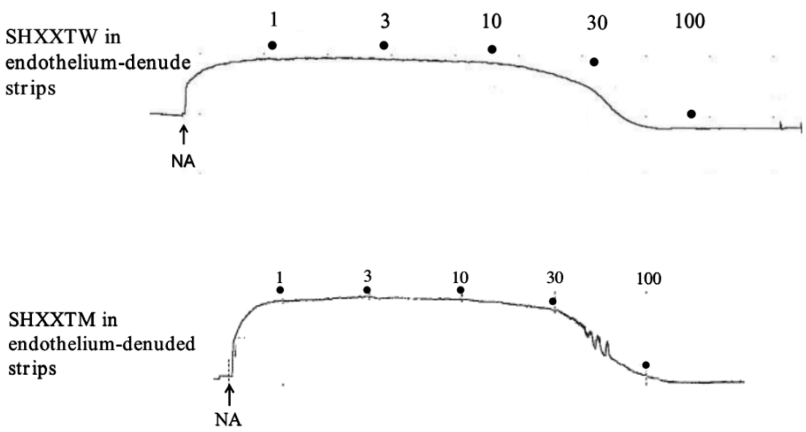


Figure 10 The SHXXTW and SHXXTM on NA-induced contractions in endothelium-denuded strips

Chapter II: The results of HPLC analysis and PCA

1. Materials and methods

1.1 Chemicals

Baicalin, baicalein, chrysophanic acid, emodin, aloe-emodin, and wogonoside were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Rhein, wogonin, palmatine chloride, coptisine chloride, and berberine chloride were purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan). Sennoside A was purchased from Sigma-Aldrich Co. LLC (Darmstadt, Germany).

Methanol and acetonitrile for sample preparation and liquid chromatography were of HPLC grade. All other chemicals and reagents were of analytical reagent grade.

1.2 Methods of HPLC

The HPLC system consisted of a Shimadzu LC-20AR Prominence liquid chromatograph pump, an SIL-10AD Prominence autosampler, an SPD-M10A Prominence diode array detector (DAD), a CTO-10ASvp column oven, and an SCL-10A Prominence communications bus module, and data were recorded by LabSolutions software (Version 5.42 SP6) (Shimadzu Co., Kyoto, Japan). Liquid chromatographic separation was achieved by using a YMC-Triart-PFP C18 column (250 mm × 4.60 mm, 5 μm) (YMC Co., Ltd., Kyoto, Japan) and column temperature was kept constant at 25 ℃. The mobile phase was composed of a mixture of acetonitrile (A) and water with 0.01 M 1-pentanesulfonic acid sodium salt plus 0.11 mL/L H_3PO_4 (B), and was delivered at the flow rate of 1 mL/min . The detection wavelength was set at 270 nm and the loading volume was 10 μL [31, 32]. All the reference standards and the SHXXT samples were dissolved in methanol and made stock solutions for detection. The gradient program is shown in Table 6.

The preparative HPLC system consisted of a Shimadzu LC-6AD liquid chromatograph pump, an SPD-10A detector (Shimadzu Co., Kyoto, Japan), and a YMC-Triart-Phenyl C18 column (250 mm × 10 mm, 5 μm) (YMC Co. Ltd., Kyoto, Japan). The mobile phase consisted of a mixture of acetonitrile (A) and water with 0.015 M ammonium formate plus 0.5% formic acid (B) (A:B = 28:72), and the flow rate was 5 mL/min. The detection wavelength was set at 270 nm and the loading volume was 0.3 mL.

Table 6 HPLC gradient program

Time (min)	A: Acetonitrile	B: 0.01M 1-Pentanesulfonic acid sodium salt plus 0.11 mL/L H_3PO_4
0	15	85
30	25	75
50	30	70
75	50	50
90	90	10
100	90	10

[*J. Nat. Med.* (2020), Table.1, https://doi.org/10.1007/s11418-019-01382-9]

1.3 Methods of PCA

PCA is a linear dimensionality reduction technique for extracting information from a high-dimensional space by projecting it into a low-dimensional sub-space. PCA preserves essential parts which have more data variation and removes non-essential parts which have less data variation. PCA is commonly used in the analysis of chromatographic data of drugs [33-36].

1.3.1 The relationship between the new principal component and the original variables

1) Each principal component is a linear combination of the original variables;
2) The number of final principal components are less than the number of original variables;
3) The final principal components contain most of the information which are contained in the original variables;
4) The first principal component should contain the most information in the original variable, followed by the second, and so on;
5) The principal components are independent of each other.

1.3.2 The mathematical model of PCA

For example, the total samples are **n**, and each sample contains variables **p**, X_1, X_2, •••, X_p, so the n ×p matrix is written as follows [37]:

$$X = \begin{bmatrix} x_{11} & x_{12} & \cdots & x_{1p} \\ x_{21} & x_{22} & \cdots & x_{2p} \\ \cdots & \cdots & \cdots & \cdots \\ x_{n1} & x_{n2} & \cdots & x_{np} \end{bmatrix} = (X_1, X_2, \cdots X_p)$$

$$X_i = \begin{bmatrix} x_{1i} \\ x_{2i} \\ \\ x_{ni} \end{bmatrix} \quad i = 1, 2, \cdots p$$

Matrix X which contains p variables is linearly transformed, and a new comprehensive variable can be formed as follows:

$$\begin{cases} f_{i1} = & a_{11}x_{i1} + a_{12}x_{i2} + \cdots & + a_{1p}x_{ip} \\ f_{i2} = & a_{21}x_{i1} + a_{22}x_{i2} + \cdots & + a_{2p}x_{ip} \\ \\ f_{ip} = & a_{p1}x_{i1} + a_{p2}x_{i2} + \cdots & + a_{pp}x_{ip} \end{cases} \quad i = 1, 2, \cdots p$$

The number k of variable is: $F_k = XA_k$, and is written in matrix F = XA

$$F = \begin{bmatrix} f_{11} & f_{12} & \cdots & f_{1p} \\ f_{21} & f_{22} & \cdots & f_{2p} \\ \cdots & \cdots & \cdots & \cdots \\ f_{n1} & f_{n2} & \cdots & f_{np} \end{bmatrix} = (F_1, F_2, \cdots F_p)$$

$$A = \begin{bmatrix} a_{11} & a_{12} & \cdots & a_{1p} \\ a_{21} & a_{22} & \cdots & a_{2p} \\ \cdots & \cdots & \cdots & \cdots \\ a_{p1} & a_{p2} & \cdots & a_{pp} \end{bmatrix} = (A_1, A_2, \cdots A_p)$$

A is considered as the principal component coefficient matrix.

This formula should conform the following conditions:

1) F_i has no relationship with F_j (i, j = 1, 2, •••, p);
2) $\sum_{i=1}^{p} a_{ij}^2 = 1$, j = 1, 2, •••, p;
3) F_1 has the biggest variance of (X_1, X_2, •••, X_p), and then F_2, F_3, •••.

Here, F_1 would be called the first principal component, F_2 would be called the second principal component, •••, F_p would be called the number p principal component.

1.3.3 The geometry model of PCA

For example, the total samples are **n**, and each sample contains **2** variables, so we could discuss the

meaning of PCA in two-dimensional.

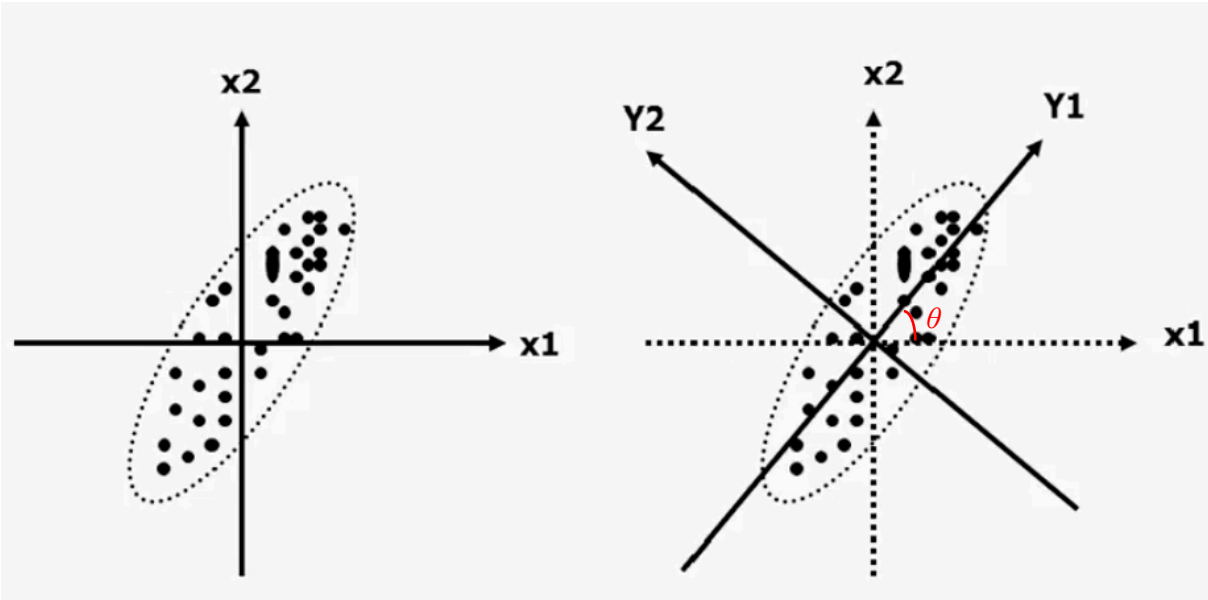


Figure 11 The geometric transformation of PCA

From the Figure 11, usually the n sample would distribute as the first graph, it looks that the samples almost have no relationship with X1 and X2. But when the axis is rotated an angle of θ, it looks that most of samples distribute along the Y1 axis, and some of them distribute along the Y2 axis, the rotation formula could be written as follows:

$$\begin{cases} Y_{1j} = X_{1j}\cos\theta + X_{2j}\sin\theta \\ Y_{2j} = X_{1j}(-\sin\theta) + X_{2j}\cos\theta \end{cases} \qquad j = 1, 2, \cdots n$$

If it is written in matrix, it would be as follows:

$$Y = \begin{bmatrix} Y_{11} & Y_{12} & \cdots & Y_{1n} \\ Y_{21} & Y_{22} & & Y_{2n} \end{bmatrix}$$

$$= \begin{bmatrix} \cos\theta & \sin\theta \\ -\sin\theta & \cos\theta \end{bmatrix} \times \begin{bmatrix} X_{11} & X_{12} & \cdots & X_{1n} \\ X_{21} & X_{22} & \cdots & X_{2n} \end{bmatrix} = U \times X$$

U is the rotation matrix and orthogonal matrix, so $U^T = 1/U$, $\sin^2\theta + \cos^2\theta = 1$. ($U^T$ is the transposed matrix of U)

Y1-Y2 has the following characters:

1) The coordinate of n in Y1 and Y2 has no relationship;

2) The variance of n in Y1 is bigger than in Y2.

Here, Y1 and Y2 would be called as the comprehensive variables of X1 and X2, Y1 would be called the first principal component, Y2 would be called the second principal component.

1.3.4 The mathematical derivation of principal component

Because of the principal components should have no relationships between each other, so the covariance matrix of principal components should be a diagonal matrix, as follows:

$$F = AX$$

So, the covariance is:

$$\mathrm{Var}(F) = \mathrm{Var}(AX) = (AX) \times (AX)^T = AXX^TA^T$$

$$= \Lambda = \begin{bmatrix} \lambda_1 & & & \\ & \lambda_2 & & \\ & & \cdots & \\ & & & \lambda_p \end{bmatrix}$$

Λ is a diagonal matrix with components λ. If the covariance matrix of original data is V, so V would be equal to correlation matrix R, $V = R = XX^T$, then, $\mathrm{Var}(F) = AXX^TA^T = ARA^T = \Lambda$, so $RA^T = A^T\Lambda$

$$\begin{bmatrix} r_{11} & r_{12} & \cdots & r_{1p} \\ r_{21} & r_{22} & \cdots & r_{2p} \\ \cdots & \cdots & \cdots & \cdots \\ r_{p1} & r_{p2} & \cdots & r_{pp} \end{bmatrix} \times \begin{bmatrix} a_{11} & a_{21} & \cdots & a_{p1} \\ a_{12} & a_{22} & \cdots & a_{p2} \\ \cdots & \cdots & \cdots & \cdots \\ a_{1p} & a_{2p} & \cdots & a_{pp} \end{bmatrix} =$$

$$\begin{bmatrix} a_{11} & a_{21} & \cdots & a_{p1} \\ a_{12} & a_{22} & \cdots & a_{p2} \\ \cdots & \cdots & \cdots & \cdots \\ a_{1p} & a_{2p} & \cdots & a_{pp} \end{bmatrix} \times \begin{bmatrix} \lambda_1 & & & \\ & \lambda_2 & & \\ & & \cdots & \\ & & & \lambda_p \end{bmatrix}$$

Expand the formula, here only the first line is shown:

$$\begin{cases} (r_{11} - \lambda_1)a_{11} + r_{12}a_{12} + \cdots + r_{1p}a_{1p} = 0 \\ r_{21}a_{11} + (r_{22} - \lambda_1)a_{12} + \cdots + r_{2p}a_{1p} = 0 \\ \\ r_{p1}a_{11} + r_{p2}a_{12} + \cdots + (r_{pp} - \lambda_1)a_{1p} = 0 \end{cases}$$

In order to find the solution to this homogeneous equation, the coefficient matrix of the equation is needed to be equal to 0, it shows as:

$$\begin{vmatrix} r_{11}-\lambda_1 & r_{12} & \cdots & r_{1p} \\ r_{21} & r_{22}-\lambda_1 & & r_{2p} \\ \cdots & \cdots & \cdots & \cdots \\ r_{p1} & r_{p2} & \cdots & r_{pp}-\lambda_1 \end{vmatrix} = 0$$

So, $|R - \lambda_1 I| = 0$, λ_1 is called the characteristic value, $a_1 = (a_{11}, a_{12}, \cdots a_{1p})$ is called the characteristic vector. From the line 2, 3, •••, we will get λ_i (i = 1, 2, •••p), also the same as a.

If we get the number p of characteristic value, $\lambda_1 \geqq \lambda_2 \geqq \cdots \geqq \lambda_p$, the characteristic vector is a_p, so

$$A = \begin{bmatrix} a_{11} & a_{12} & \cdots & a_{1p} \\ a_{21} & a_{22} & \cdots & a_{2p} \\ \cdots & \cdots & \cdots & \cdots \\ a_{p1} & a_{p2} & \cdots & a_{pp} \end{bmatrix} = \begin{bmatrix} a_1 \\ a_2 \\ \cdots \\ a_p \end{bmatrix}$$

The covariance of F_1, $Var(F_1) = a_1XX^Ta_1^T = a_1Ra_1^T = \lambda_1$, and $Var(F_i) = \lambda_i$, the variance is reduced gradually.

$Cov(a_i^T X^T, a_jX) = a_i^T Ra_j = a_i(\sum_{a=1}^{p} \lambda a\ a_a a_a^T)a_j = \sum_{i=1}^{p} \lambda a\ (a_i^T a_a)(a_a^T a_j) = 0$, $i \neq j$. These process indicates that the covariance matrix is a diagonal matrix, and the value of the diagonal line is equal to the characteristic value, the value of A is as the characteristic vector. From these process, the principal components were written as follows:

$$\begin{cases} F_1 = a_{11}x_1 + a_{12}x_2 + \cdots + a_{1p}x_p \\ F_2 = a_{21}x_1 + a_{22}x_2 + \cdots + a_{2p}x_p \\ \\ F_p = a_{p1}x_1 + a_{p2}x_2 + \cdots + a_{pp}x_p \end{cases}$$

1.3.5 The mathematical calculation process of PCA

For example, the sample matrix is:

$$X = \begin{bmatrix} x_{11} & x_{12} & \cdots & x_{1p} \\ x_{21} & x_{22} & \cdots & x_{2p} \\ \cdots & \cdots & \cdots & \cdots \\ x_{n1} & x_{n2} & \cdots & x_{np} \end{bmatrix}$$

1) First step, standardize the raw data:

$$x_{ij}^{*} = (x_{ij} - \overline{x_j}) / \sqrt{var(x_j)} \quad (i = 1, 2, \cdots n; j = 1, 2, \cdots p)$$

$$\overline{x_j} = (1/n) \times \sum_{i=1}^{n} x_{ij}, \; var(x_j) = [1/(n-1)] \times \sum_{i=1}^{n} (\overline{x_{ij}} - x_j)^2 \; (j = 1, 2, \cdots p)$$

2) Coefficient calculation:

$$R = \begin{bmatrix} r_{11} & r_{12} & \cdots & r_{1p} \\ r_{21} & r_{22} & \cdots & r_{2p} \\ \cdots & \cdots & \cdots & \cdots \\ r_{p1} & r_{p2} & \cdots & r_{pp} \end{bmatrix}$$

$$r_{ij} = Cov(x_i, x_j) / (\sqrt{D(x_i)} \times \sqrt{D(x_j)}) \quad (i, j = 1, 2, \cdots p)$$

3) Calculation of characteristic value λ_p ($\lambda_1, \lambda_2, \cdots \lambda_p$), and characteristic vector $a_i = (a_{i1}, a_{i2}, \cdots a_{ip})$, $i = 1, 2, \cdots p$.

4) Choose the principal component which has the biggest variance, and calculate the contribution (α) of principal component, then choose the number of principal component. Usually, if the contribution of ($\alpha_1 + \alpha_2 + \cdots \alpha_p$) is around 80%, it indicates that these principal components contain most of the information of the raw data.

$$\alpha = \lambda_i / \sum_{i=1}^{p} \lambda_i \; (i = 1, 2, \cdots p)$$

5) Calculate the score of samples in principal components.

$$F = \begin{bmatrix} f_{11} & f_{12} & \cdots & f_{1p} \\ f_{21} & f_{22} & \cdots & f_{2p} \\ \cdots & \cdots & \cdots & \cdots \\ f_{n1} & f_{n2} & \cdots & f_{np} \end{bmatrix} = (F_1, F_2, \cdots F_p)$$

The final score of each sample is $F = a_1F_1 + a_2F_2 + \cdots + a_pF_p$.

1.4 Methods of PCR

Principal component regression (PCR) is a regression analysis technique that is based on principal component analysis (PCA). Usually, PCA is used for estimating the unknown regression coefficients in a standard linear regression model. While in PCR, instead of regressing the dependent variable on the explanatory variables directly, the principal components of the explanatory variables are used as regressors. One typically uses only a subset of all the principal components for regression, making PCR a kind of regularized procedure and also a type of shrinkage estimator [38-40].

The main idea with principal component regression is to replace the X into F (F_1, F_2, ••• F_p), F is obtained as the principal components (PCs) from the PCA of the predictor data.

Multiple linear regression (MLR) is: X to Y,

Principal component regression is X to F, F to Y (in our study, Y is confined as EC_{50}).

$$X \xrightarrow{\text{MLR}} Y \qquad\qquad X \xrightarrow{\text{PCA}} F \xrightarrow{\text{MLR}} Y$$

In the PCA, F is calculated by the following formula:

$$F_1 = a_{11}x_1 + a_{12}x_2 + \cdots + a_{1p}x_p$$

$$F_2 = a_{21}x_1 + a_{22}x_2 + \cdots + a_{2p}x_p$$

$$F_p = a_{p1}x_1 + a_{p2}x_2 + \cdots + a_{pp}x_p$$

So, usually $Y = t + t_1 F_1 + t_2 F_2 + \cdots + t_p F_p$, but as mentioned in section **1.3**, when ($F_1 + F_2$) is around 80%, ($F_1 + F_2$) could replace all the sample information, so Y would be $Y = t + t_1 F_1 + t_2 F_2$, also $Y = t + t_1 (a_{11}x_1 + a_{12}x_2 + \cdots + a_{1p}x_p) + t_2 (a_{21}x_1 + a_{22}x_2 + \cdots + a_{2p}x_p)$, this is the calculation process, in this study, the results were calculated by the same methods.

1.5 Statistical analysis

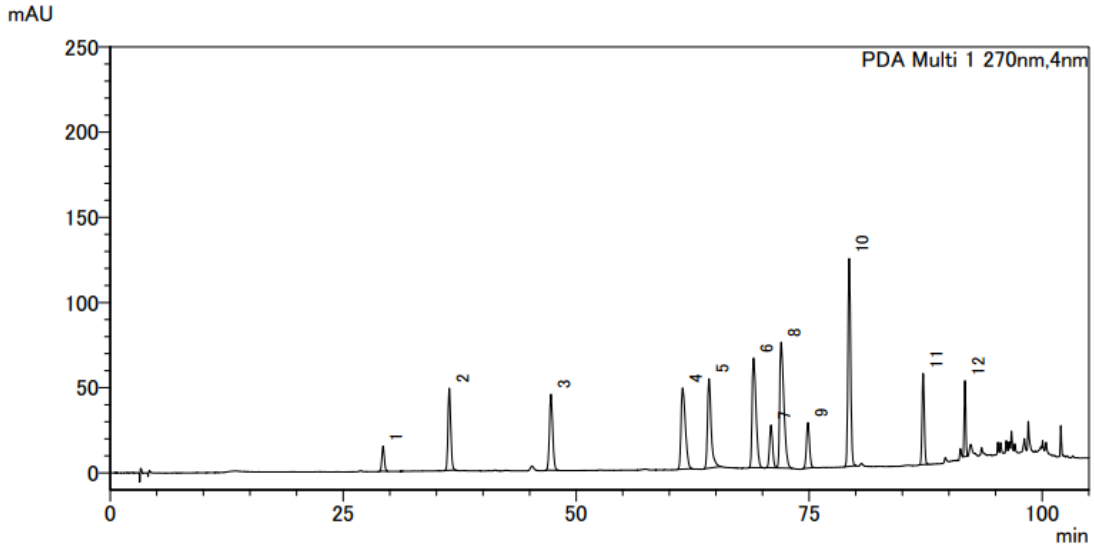
Data were expressed as means ± S.E.M. Significant differences between groups were assessed by one-way analysis of variance (1-ANOVA) followed by Dunnett's method. A P-value less than 0.05 was considered significant.

2. Results

2.1 Results of HPLC analysis

Total 39 peaks were detected in the SHXXTM, HPLC chromatogram was shown in Figure 12. Among of them, 11 compounds were identified compared with the reference standards (Table 7), and the amounts of baicalin, berberine, wogonoside, coptisine, baicalein, and palmatine were higher than the others. At the same time, the amounts of baicalin, berberine, baicalein, and palmatine in SHXXTM were measured and the results were shown in Table 8. All samples for PCA were also analyzed by HPLC and the samples were found to have different peak distributions. The HPLC data were described in Figure S2 (Figure S2 is shown in supplementary materials).

a:



b:

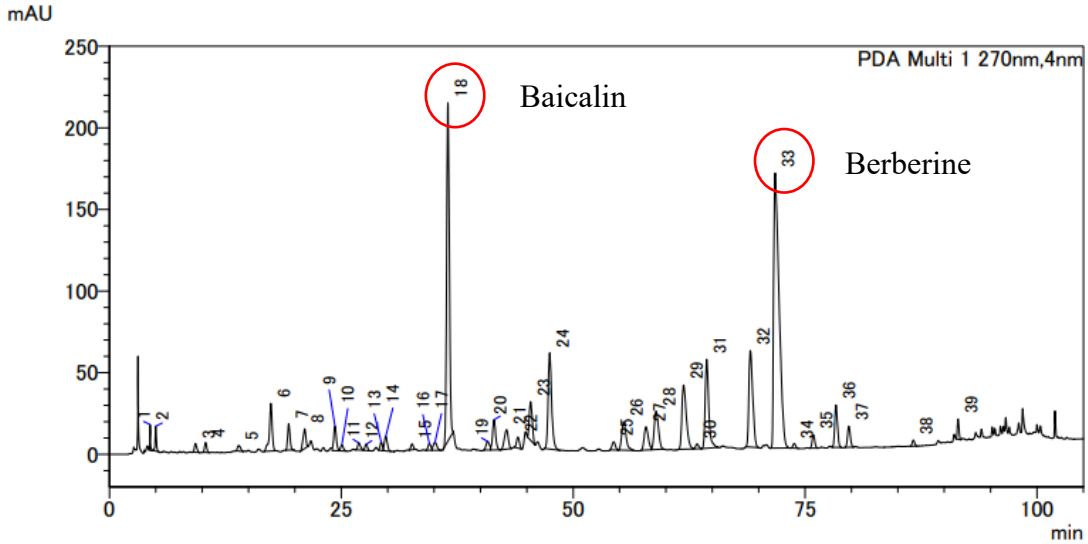


Figure 12

a: HPLC chromatogram of Reference standards and

b: HPLC chromatogram of SHXXTM

[*J. Nat. Med.* (2020), Fig.1, https://doi.org/10.1007/s11418-019-01382-9]

Table 7 The corresponding peak in reference standards and SHXXTM

Name	RS. No	SHXXTM. No
Senoside A	1	14
Bacalin	2	18
Wogonoside	3	24
Coptisine	4	29
Baicalein	5	31
Palmatine	6	32
Aloe-emodin	7	-
Berberine	8	33
Rhein	9	34
Wogonin	10	37
Emodin	11	38
Chrysopanic acid	12	39

Table 8 Amounts of baicalin, berberine, baicalein, and palmatine in SHXXTM

Compound	Amount (mg / 100 mg)
Baicalin	8.0
Berberine	6.6
Baicalein	2.0
Palmatine	1.8

[*J. Nat. Med.* (2020), Table 3, https://doi.org/10.1007/s11418-019-01382-9]

2.2 Results of PCA analysis

From the results of HPLC, 39 peaks were totally detected with different retention times of 28 samples. Each peak area of their peaks was calculated for every samples and saved in a single excel to form a 2D data matrix with dimensions of 28 samples (objects) × 39 peaks (variables) (Figure S3 is shown in supplementary materials). These data were imported to multivariate data analysis "The Unscrambler® X" software (Camo Analytics Co., Oslo, Norway) for PCA. The results are shown in Figures 13, 14, and S4 (Figure S4 is shown in supplementary materials).

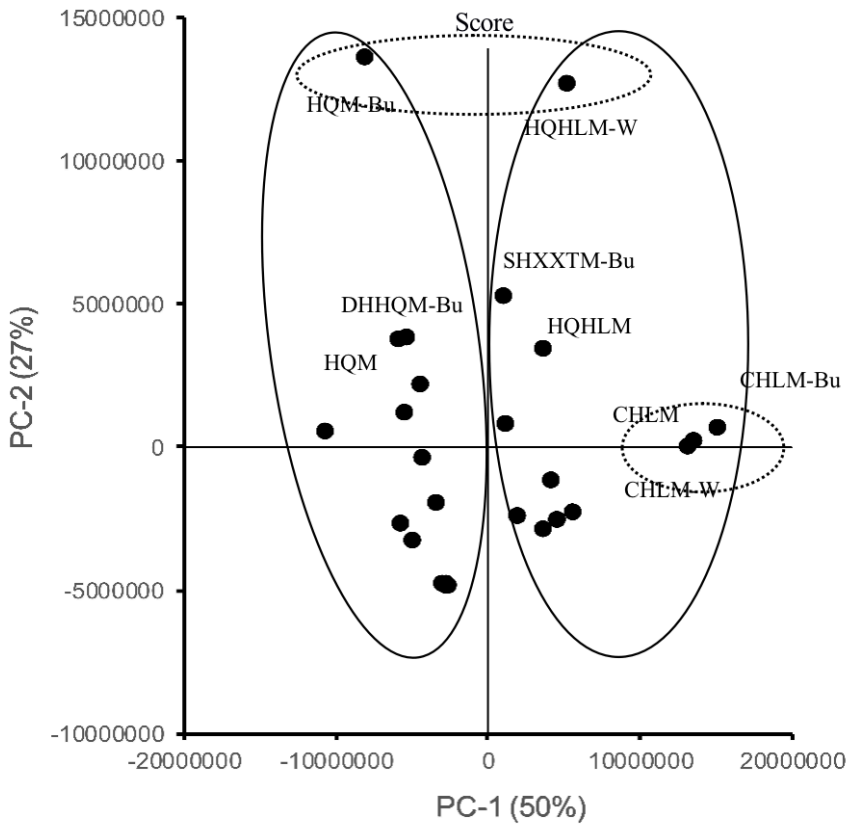


Figure 13 Scores of all samples by PCA

[*J. Nat. Med.* (2020), Fig.4, https://doi.org/10.1007/s11418-019-01382-9]

PCA was used to clearly visualize the difference of the extracts among 28 samples. Usually, PCA calculation could be continued until 39 principal components (PCs) have been obtained. However, in this study, the PCA resulted in a model in which the first two PCs extracted 77% of the total chromatogram variation. The first PC (PC-1) and second PC (PC-2) accounted for 50 and 27 % of the total chromatogram variation, respectively, so that it indicated that the first two principal components (PCs) could summarize 77% of the HPLC information [33, 34]. Therefore, the PCA results for PC-1 and PC-2 were considered below.

The projections of the points from the original variable spaces on a PC axis are called the Scores of the objects. Based on the PCA calculation, the Score-1 and Score-2 values of all samples were plotted in the two dimensions of PC-1 and PC-2 axis, and were depicted in Figure 13. Score plot showed two groups largely apart from the origin (0, 0) on the PC-1 and PC-2 axis. On PC-1 axis, three samples, CHL methanol extract (CHLM), the water fraction of CHLM (CHLM-W), the *n*-butanol fraction of CHLM (CHLM-Bu), had the higher Score-1 values than the other samples. Meanwhile, two samples, the *n*-butanol fraction of HQ methanol extract (HQM-Bu), the water fraction of HQ and HL methanol extract (HQHLM-W), had the higher Score-2 values on the PC-2 axis. These results indicated that these samples might contain important components for our study.

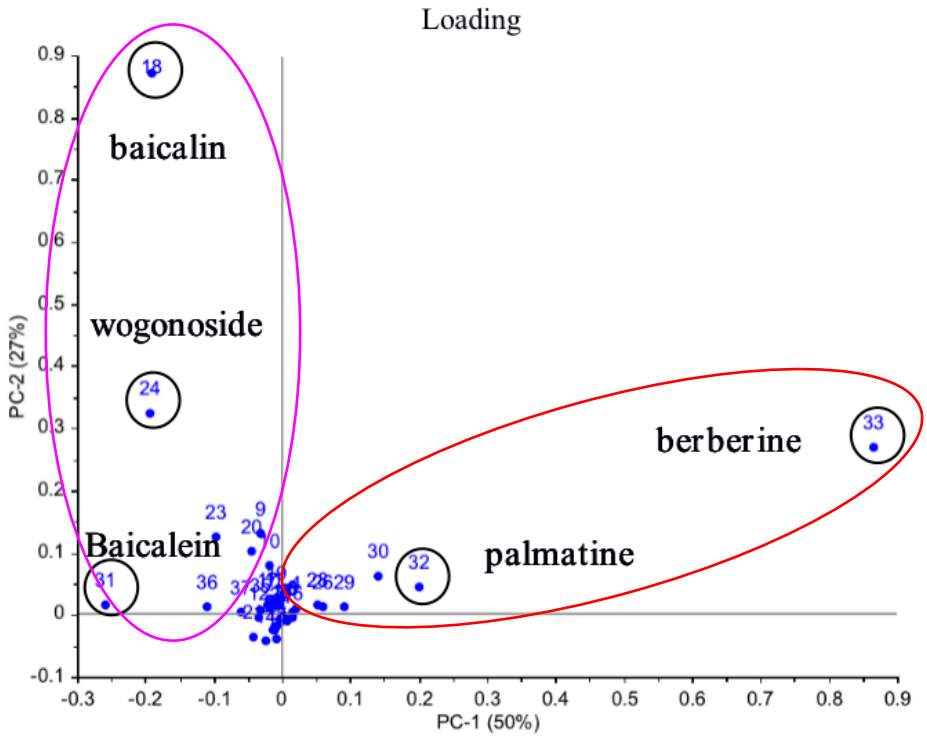


Figure 14 The Loadings about variables of samples

[*J. Nat. Med.* (2020), Fig.4, https://doi.org/10.1007/s11418-019-01382-9]

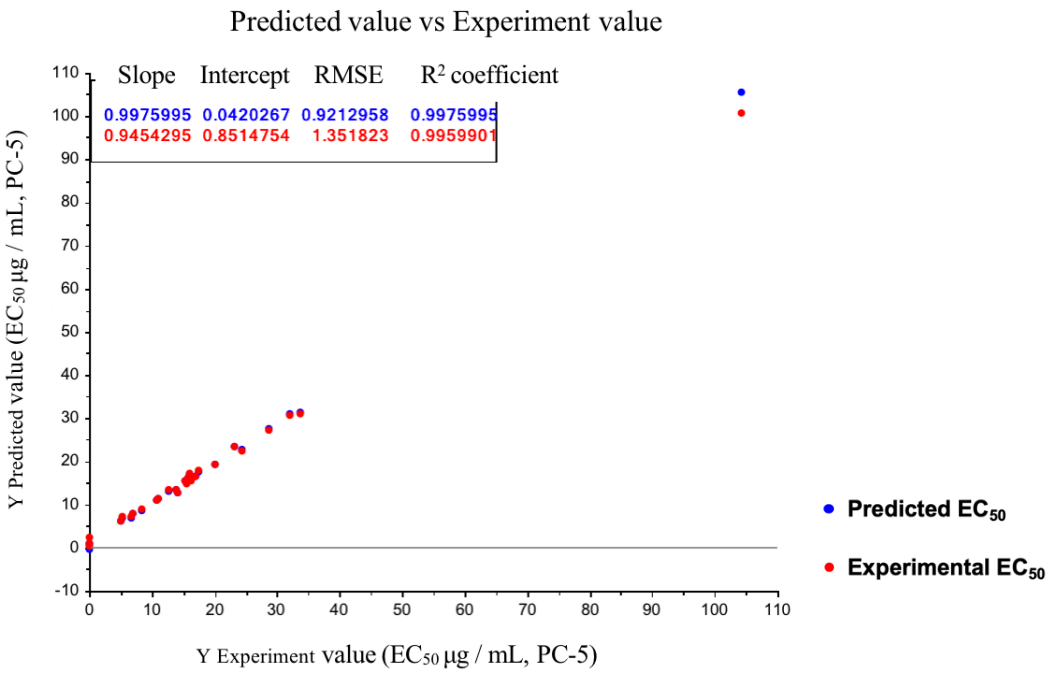
The Score values of the PCs are the weighted sums of the original variables and the weights contain useful information about the variables [41]. These weights are called Loadings and can reveal the variables that are responsible for the main variation in the data. To elucidate the contribution of each original variable to Score-1 and Score-2 values, the Loading plot on the PC-1 and PC-2 axis was depicted in Figure 14. On the PC-1 axis, the peak No. 33 (berberine) and No. 32 (palmatine) had the most positive Loading-1 factor, while the peak No. 18 (baicalin), No. 24 (wogonoside) and No. 31 (baicalein) had the negative Loading-1 factors. This indicated that the samples which got the higher Score-1 values in Figure 13 have higher contents of berberine and palmatine, and lower contents of baicalin, wogonoside and baicalein. In fact, three samples, CHLM, CHLM-W and CHLM-Bu, showed larger peak areas of No. 33 and No. 32 than the other samples, and almost no peak No. 18, No. 24, and No. 31 (Figure S2 is shown in supplementary materials). On the other hand, on the PC-2 axis, although most variables showed positive Loading-2 factors, three of the variables, peak No. 18 (baicalin), No. 24 (wogonoside) and No. 33 (berberine), were comparatively lager in the Loading-2 factor than other variables and the values were in the order of peak No. 18 >> No. 24 > No. 33. In fact, two samples, HQM-Bu and HQHLM-W, showed larger peak area of peak No. 18 (baicalin) than the other samples. However, these two samples had opposite Score-1 values, i.e., HQM-Bu had a negative Score-1 value, while HQHLM-W had a positive Score-1 value, respectively. This might due to that HQHLM-W contained peak No. 33 (berberine) while HQM-Bu did not.

From these results, the peak No. 33 (berberine) and No. 32 (palmatine) showed high contribution to PC-1, while the peak No. 18 (baicalin), No. 24 (wogonoside) and No. 33 (berberine) showed high contribution to PC-2, the peak No. 31 (baicalein) had the negative Loading-1 factor and positive Loading-2 factor, and was far from the origin (0, 0), so we considered it might also have a contribution. Finally, we preliminary speculated that these five peaks might have important role in our research.

2.3 Results of PCR analysis

Connected the PCA results and EC_{50} data of all samples, we used principal component regression (PCR) analysis to calculate whether the results were reliable or not. The results showed that the values of predicated EC_{50} and experimentally observed EC_{50} were almost same, and regression coefficients was larger than 0.99. It showed a good linearity in the results when calculated by the principal components [40, 42, 43] (Figure 15, whole calculation graphs are shown in Figure S5 in supplementary materilas).

PCR analysis Area (%)



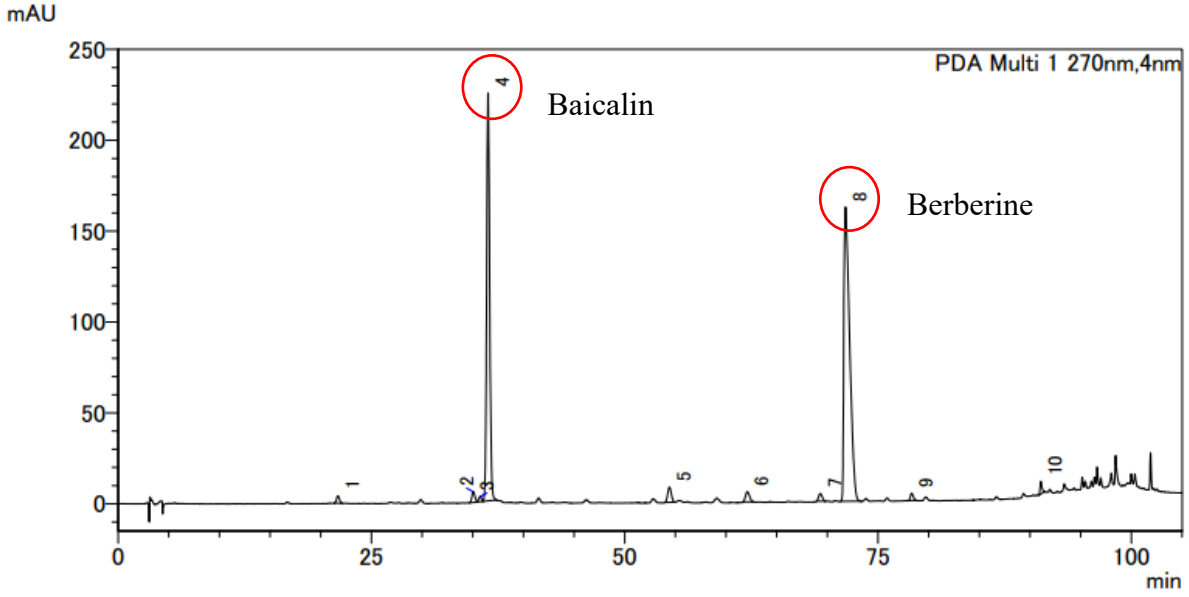
RMSE: root-mean-square error

Figure 15 The results of PCR

2.4 Results of preparative HPLC

As described in section 2.2, PCA results indicated that baicalin, berberine, palmatine, baicalein, and wogonoside markedly contributed to the pharmacological activity. Connected the HPLC data in section 2.1, the HPLC chromatogram revealed that the amounts of baicalin and berberine were relatively higher than those of the other compounds. Considered to minimize the losses as much as possible, preparative HPLC was used to fractionate SHXXTM into the baicalin and berberine part (SHXXTM-PHPLC-BB) and SHXXTM except baicalin and berberine part (SHXXTM-PHPLC-except BB). The chromatograms of the two parts are shown in Figure 16.

a:



b:

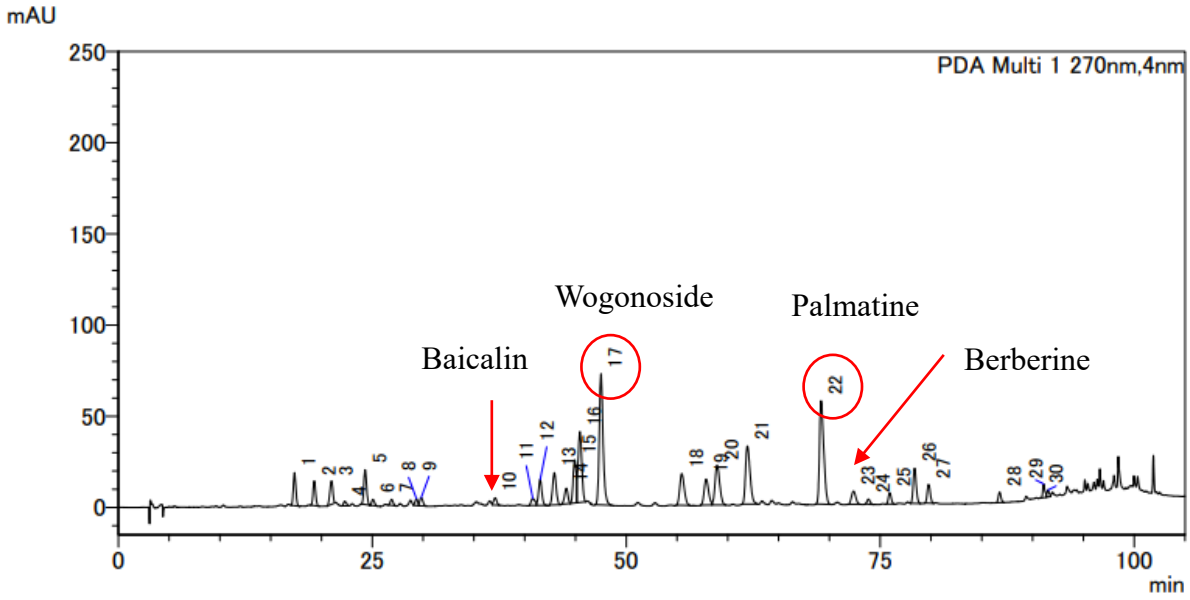


Figure 16

a: HPLC chromatogram of SHXXTM baicalin and berberine part (SHXXTM-PHPLC-BB)

b: HPLC chromatogram of SHXXTM except baicalin and berberine part (SHXXTM-PHPLC-except BB)

[*J. Nat. Med.* (2020), Fig.2, https://doi.org/10.1007/s11418-019-01382-9]

Meanwhile, the two parts obtained by preparative HPLC were also tested on the *in vitro* experiments for comparsion with SHXXTM, and the results are shown in Table 9 and Figure 17. The results indicated

that the baicalin and berberine part almost had the same effect compared to SHXXTM.

Table 9 EC_{50} of SHXXTM-PHPLC-BB and SHXXTM-PHPLC-except BB

Sample Name	**EC_{50} (μg/ml)**
SHXXTM	16.2±1.1
SHXXT-PHPLC-BB	10.5±0.1
SHXXT-PHPLC-except BB	>100

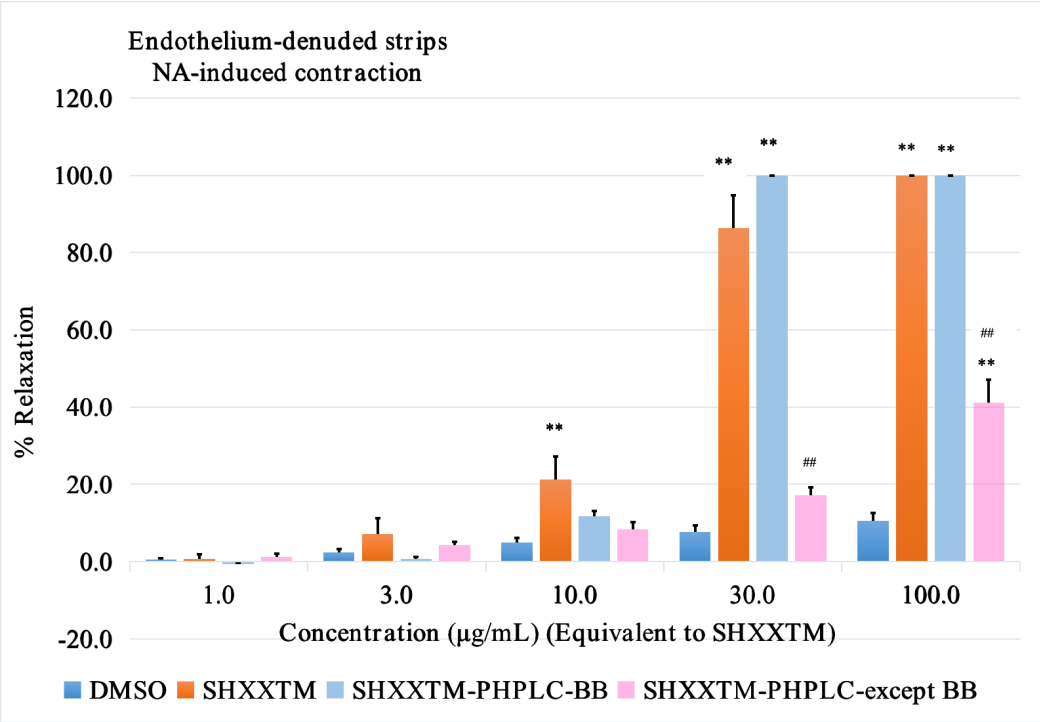


Figure 17 Vasorelaxant effects of SHXXTM, SHXXTM-PHPLC-BB and SHXXTM-PHPLC-except BB on NA -induced contractions in endothelium-denuded strips

Each bar graph represents the mean with S.E.M (n = 4–6), $^{*}p < 0.05$, $^{**}p < 0.01$ vs. DMSO control group and $^{\#\#}p < 0.01$ vs. SHXXT group.

[*J. Nat. Med.* (2020), Fig.5, https://doi.org/10.1007/s11418-019-01382-9]

2.5 Results of baicalin, berberine, palmatine, baicalein, and their combinations on NA-induced contractions in endothelium-denuded strips

To further verify that the combination of baicalin and berberine might have the same effect as SHXXTM, here the reference standards of baicalin, berberine, palmatine and baicalein were used in the *in vitro* antivascular contraction experiments.

In the *in vitro* antivascular contraction experiments, SHXXTM at the concentration of 100 mg/mL

was used (final maximum concentration in the medium: 100 μg/mL). As shown in Table 8, HPLC analysis demonstrated that 100 mg of SHXXTM contained 8.0 mg of baicalin, 6.6 mg of berberine, 2.0 mg of baicalein, 1.8 mg of palmatine, and 81.6 mg of the other compounds. Therefore, the reference standards of baicalin, berberine, baicalein, and palmatine, also their combinations (the concentrations of them were equal to the concentrations of SHXXTM) were used to compare and verify the effects on the *in vitro* experiments. The results are shown in Table 10.

Table 10 Vasorelaxant effects of baicalin, berberine, palmatine, baicalein, and their combinations on NA-induced contractions in endothelium-denuded strips

Sample name	**Final maximum concentration in medium (μg/mL)**				
	1.0	3.0	10.0	30.0	100.0
DMSO	0.1±0.3	2.3±0.8	5.0±1.2	7.7±1.8	10.5±2.0
SHXXTM	0.6±1.2	7.1±4.1	21.2±6.0	86.3±8.6**	100.0±0.0**
Baicalin 8.0 mg	2.9±0.9	5.0±1.7	13.9±4.5	100.0±0.0**	100.0±0.0**
Berberine 6.6 mg	0.0±0.3	1.0±0.5	10.7±1.6	34.3±3.8**##	53.6±4.0**##
Baicalin+Berberine (8.0+6.6) mg	2.9±1.0	6.9±1.2	32.1±8.5**	100.0±0.0**	100.0±0.0**
Baicalin+Berberine+Palmatine (8.0+6.6+1.8) mg	5.1±1.0	15.0±3.2	34.7±5.0**	100.0±0.0**	100.0±0.0**
Baicalin+Berberine+Palmatine+Baicalein (8.0+6.6+1.8+2.0) μg/mL	2.2±0.9	4.3±1.3	20.4±5.8	100.0±0.0**	100.0±0.0**

Each value represents the mean ± S.E.M. (n = 4–6). Final concentration in medium was equivalent to SHXXTM. **p<0.01 vs. DMSO control group and ##p<0.01 vs. SHXXTM group.
[*J. Nat. Med.* (2020), Fig.5, https://doi.org/10.1007/s11418-019-01382-9]

From the results, it indicated that the BB combination (reference standards) also had the same effects compared to SHXXTM. At the same time, according to the literature [44], the theoretic synergism effects of BB combination was calculated (Table 11). The theoretic $EC_{50} = 2 \times XY / (X + Y) = 29.1$ was larger than the experimental EC_{50}, suggesting that the BB combination had the synergism effects.

Table 11 Synergism effects of baicalin and berberine

Sample Name	**EC_{50} (μg/mL)**
Baicalin 8.0 mg	18.4±1.2
Berberine 6.6 mg	69.1±3.3
Baicalin+Berberine (8.0+6.6) mg	15.3±0.9
Theoretic	29.1

3. Discussion

PCA is a linear dimensionality reduction technique for extracting information from a high-dimensional space by projecting it into a low-dimensional sub-space. PCA preserves essential parts which have more data variation and removes non-essential parts which have less data variation. Traditional prescriptions and medicines usually contain a lot of compounds, which make it difficult for researchers to carry out quality control and elucidate the underlying mechanism. For this reason, many researchers would like to use some methods such as prescription disassembling, HPLC fingerprint, prescription ingredients combination, *in vitro* pharmacological experiments, *in vivo* metabolites experiments to take detailed research about them [45-47]. In this study, these methods were also used.

Various data were needed for the PCA to observe the relationship between the peaks, so here, water, *n*-butanol, and ethyl acetate were used to fractionate SHXXTM, because they had the different polarity from each other, so the compounds would have a different distribution in them, which would cause the different pharmacological activity. The other solvents also could be used to add the total numbers of fractions, which might be made the results more accuracy and reliable.

PCA results showed that baicalin, berberine, palmatine, baicalein and wogonoside contributed significantly to the vasorelaxant effect. Two samples, HQM-Bu and HQHLM-W, had higher Score-2 values in PCA calculation, and also showed lower EC_{50} values of 5.2 and 5.0 μg/mL, respectively, than other samples. Since HQM-Bu did not contain berberine, it was indicated that baicalin in two samples had high contribution to the pharmacological activity. The *n*-butanol fraction of SHXXTM (SHXXTM-Bu), HQ and CHL methanol extract (HQHLM) also showed comparatively low EC_{50} values of 6.7 and 6.9 μg/mL, respectively, although their values were lower than those of HQM-Bu and HQHLM-W. These samples contained baicalin and berberine more rich than other samples except HQM-Bu and HQHLM-W. Meanwhile, HQ methanol extract (HQM) and the *n*-butanol fraction of DH and HQ methanol extract (DHHQM-Bu) had almost same Score-2 value with SHXXTM-Bu and HQHLM. The two samples had EC_{50} values of 15.8 (HQM) and 17.0 μg/mL (DHHQM-Bu), respectively, and showed lower potency than SHXXTM-Bu and HQHLM. Comparing with SHXXTM-Bu and HQHLM sample, HQM and DHHQM-Bu contained the almost same baicalin content and higher wogonoside but did not contain berberine. Therefore, this result indicated that berberine also might contributed to the pharmacological activity, although its potency was weaker than that of baicalin. Meanwhile, three samples, CHLM, CHLM-W and CHLM-Bu, with high Score-1 value, and EC_{50} values between two samples, SHXXTM-Bu and HQHLM, and two samples, HQM and DHHQM-Bu, e.g., 8.3 (CHLM-W), 10.8 (CHLM) and 11.0 μg/mL (CHLM-Bu), respectively. These samples contained berberine more rich than the other samples. Therefore, this result supported the above consideration that berberine also contributed to the pharmacological activity. In addition, three samples were also contained high palmatine contents. Therefore, palmatine also might contribute to the pharmacological activity with

berberine. Besides these, although baicalein was got a low value in Score-2, but it was far from the origin, and *in vivo* baicalin would change into baicalein, so here we considered that baicalein might get a weak contribution. On the other hand, the samples localized around the origin (0, 0) in Score plot showed low or no potency to the pharmacological activity.

Meanwhile, from the results of *in vitro* antivascular contraction experiments, it was found that SHXXTM-PHPLC-BB and the BB combination (reference standards) had almost the same effects as SHXXTM, whereas the vasorelaxant effects of SHXXTM-PHPLC-except BB were obviously decreased. It indicated that BB combination could replace the SHXXT in the *in vitro* study, also it confirmed that the process from prescription disassembling to PCA analysis is suitable for prescription research, and PCA is an accurate and reliable tool for scientific research of traditional medicines. Besides this, it was found that the combination of baicalin and berberine might have synergism effects on the *in vitro* study, so this result might be important for the *in vivo* study.

Chapter III: *In vitro* mechanism research and *in vivo* study

1. Materials and methods

1.1 Materials

The three crude drugs, DH, HQ, and CHL, used in this study, were purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan). The lot numbers of DH, HQ, and CHL used in this study are 007016001, 001116002, and 001317001, respectively, as described in Chapter I.

1.2 Chemicals

Baicalin, glibenclamide and formaldehyde were purchased from TCI Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Berberine chloride, 4-Aminopyridine (4-AP), tetraethylammonium chloride (TEA), barium chloride dihydrate (Ba^{2+}), and amlodipine were purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan). Calphostin C and NG-nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Abcam PLC (Cambridge, England). Rottlerin was purchased from Enzo Life Science, Inc. (Farmingdale, USA). Diazoxide were purchased from Sigma-Aldrich Co. LLC. (Darmstadt, Germany).

All other chemicals and reagents are of analytical reagent grade.

1.3 Animals

All procedures and protocols (No: PCOG-17-008 and PCOG-17-010) were approved by the Animal Care and Use Committee of Kyoto Pharmaceutical University. Male Sprague-Dawley rats (200–300 g, 7–8 weeks), male WKY/Izm rats (250–300 g, 10 weeks), and male SHRs (SHR/Izm, 250–300 g, 10 weeks) were housed under constant temperature and illumination conditions. The rats were allowed access to food and water ad libitum. All rats were purchased from Japan SLC, Inc. (Shizuoka, Japan).

1.4 Methods

1.4.1 The extraction of SHXXT

A blended mixture of DH, HQ, and CHL in 1:1:1 ratio was refluxed with methanol for 1.5 h, and this procedure was repeated three times. The product collected by refluxing was filtered. The filtrate was concentrated under reduced pressure at 40 ℃ to obtain the solid extract, as described in Chapter I.

1.4.2 Blood pressure measurement

SHRs were randomly divided into seven groups where each group had at least six rats. WKY rats were used as the normal group. All rats were housed for one week to adapt the environments. The initial

average SBP of SHRs used in the experiments was 180 ± 10 mmHg. The doses of the samples for oral administration are described in Table 12. Rat body weights were measured two times a week, and heart rates and blood pressures were measured once a week. SBPs of SHRs were evaluated by a noninvasive tail cuff method using BP-98A (Softron Co., Ltd., Tokyo, Japan) [17, 48].

Table 12 Oral administration samples and doses

Group	Dose
Normal (WKY rats)	0.5% CMCNa solution
Control (SHRs)	0.5% CMCNa solution
Positive (Nifedipine)	5 mg/kg/day
SHXXTM-low-dose	200 mg/kg/day
SHXXTM-middle-dose	400 mg/kg/day
SHXXTM-high-dose	800 mg/kg/day
Baicalin and Berberine	(32 + 26) mg/kg/day

[*J. Nat. Med.* (2020), Table 2, https://doi.org/10.1007/s11418-019-01382-9]

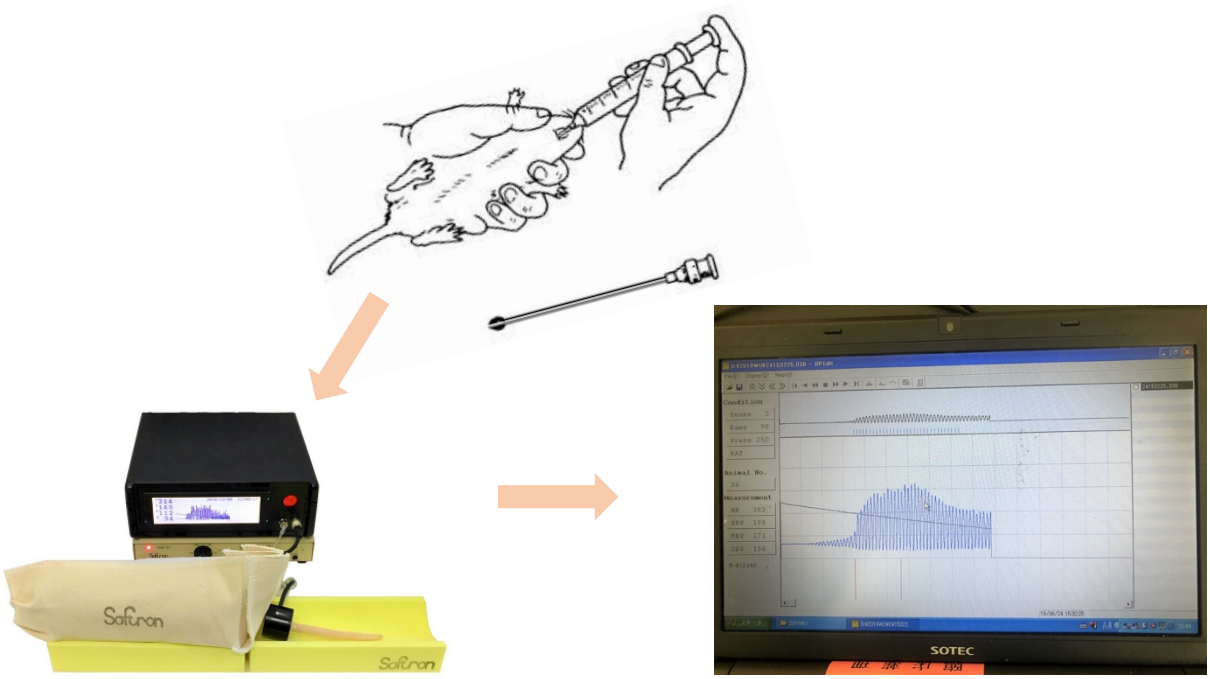


Figure 18 The process of blood pressure measurement of SHRs

1.4.3 Analysis of left ventricular hypertrophy index (LVHI), cardiac index (CI) and arota hematoxylin-eosin staining (HE) slice of SHRs

After administration for six weeks, all the rats were killed by anesthetized excessively. The hearts

were removed, rinsed with iced normal saline, and weighed. Then, the pericardial tissues were removed and the left ventricle was separated along the ventricular septum. Saline was absorbed by filter paper and the left ventricle was weighed. LVHI was defined by Formula 1 and CI was defined by Formula 2 [49, 50].

Formula 1 LVHI = left ventricle (mg)/body weight (g)

Formula 2 CI = heart weight (g)/body weight (g) x 100

The aortas of rats were separated and fixed in formalin solution for pathological section observation. Briefly, rat aorta was fixed with 4% formaldehyde. And the hematoxylin and eosin (H&E)-stained samples for microscope were prepared (CiteMed Co., Ltd., Kyoto, Japan). Briefly, the fixed tissues were dehydrated in a serial ethanol solution, hyalinized in xylene and embedded in molten paraffin at 62 ℃ overnight. Blocks were cut into 4 μm sections, which were then stained with H&E [50, 51].

1.4.4 Tissue preparation and flow chart of *in vitro* antivascular experiments

Rat thoracic aorta were carefully removed and cut into 2–3 mm long rings with endothelium or helical strips without endothelium (*ca*. 2 mm wide x 15 mm long). The rings and strips preparations were same as previous description (section **1.4.2** of **Chapter I**). In the presence of different activators or inhibitors, all the activators or inhibitors were added before the second contraction and maintained for 15 minutes and then High K^+ and NA were added to induce the second contraction. After that, the test samples were cumulatively added into the bath. Here, the test sample is the combination of baicalin and berberine (8.0 + 6.6) mg/mL (the concentrations of them were equal to the concentrations of SHXXTM).

1.5 Statistical analysis

Data were expressed as means ± S.E.M. Significant differences between the groups were assessed by one-way analysis of variance (1-ANOVA) followed by the Dunnett's method. A P-value less than 0.05 was considered significant.

2. Results

2.1 Antihypertensive effects of SHXXTM and BB combination in SHRs

All SHRs were subjected to the procedures described in section **1.4.2** of **Chapter III** and the results are shown in Table 13. As shown in Table 13, from the fourth week onward, the SHXXTM-low-dose group, the SHXXTM-middle-dose group, the SHXXTM-high-dose group, and the BB combination group significantly reduced the increase in rate of SBP compared to the control group. In the BB group, of which the BB contents were equivalent to the SHXXTM-middle-dose group, almost the same effects were observed as compared to those of the SHXXTM-middle-dose group.

Table 13 Effects of various doses of SHXXTM and BB on SHRs systolic pressure

Group	Systolic Pressure（mmHg）(Mean ± S.E.M.)						
	11 weeks (start)	12 weeks	13 weeks	14 weeks	15 weeks	16 weeks	17 weeks
Normal	114.6±2.2**	121.7±2.9**	125.1±2.9**	122.7±3.4**	127.0±2.0**	126.5±2.3**	126.6±2.6**
Control	179.0±1.8	183.7±2.2	184.7±1.4	193.0±2.8	199.4±1.5	204.4±1.5	210.6±1.8
Positive (Nifedipine)	178.3±1.1	144.3±5.3**	138.4±1.4**	142.4±4.1**	144.2±5.0**	130.1±4.1**	133.1±3.1**
SHXXTM-low-dose	176.5±1.9	176.2±2.4	183.3±3.2	187.5±2.1	**188.4±0.8***	**192.4±1.2****	**195.3±2.0****
SHXXTM-middle-dose	181.3±2.5	180.1±1.8	179.8±1.8	184.9±2.4	**186.0±1.9****	**187.1±2.0****	**190.0±1.4****
SHXXTM-high-dose	176.2±0.9	183.1±5.1	179.6±2.3	182.2±3.7	**184.0±3.7****	**181.0±2.2****	**179.0±1.5****
Baicalin & Berberine	180.8±2.1	175.0±1.7	178.0±1.6	185.0±1.4	**185.9±1.0****	**190.4±2.2****	**190.7±2.5****

Each value represents the mean ± S.E.M. (n = 6–10). Asterisks denote significant difference from control group, *$p < 0.05$, **$p < 0.01$.

[*J. Nat. Med.* (2020), Table 5, https://doi.org/10.1007/s11418-019-01382-9]

2.2 Results of LVHI and CI

The results of LVHI and CI of different oral administration groups are shown in Table 14. The LVHI values showed significant difference, whereas the CI values showed no change.

Table 14 Effects of SHXXTM and the BB combination on LVHI and CI in SHRs

Group	LVHI	CI
Normal (WKY rat)	0.84 ± 0.07**	0.29 ± 0.01**
Control (SHR)	1.57 ± 0.05	0.38 ± 0.00
Nifedipine (5 mg/kg/d)	1.16 ± 0.05**	0.37 ± 0.01
SHXXTM-low-dose (200 mg/kg/d)	1.59 ± 0.03	0.37 ± 0.01
SHXXTM-middle-dose (400 mg/kg/d)	1.22 ± 0.02**	0.38 ± 0.00
SHXXTM-high-dose (800 mg/kg/d)	1.19 ± 0.03**	0.36 ± 0.01
BB (32 + 26 mg/kg/d)	1.24 ± 0.02**	0.37 ± 0.02

Each value represents the mean ± S.E.M. (n = 4–6). Asterisks denote significant difference from control group, **$p < 0.01$.

[*J. Nat. Med.* (2020), Table 1, https://doi.org/10.1007/s11418-020-01387-9]

2.3 Results of the photograph of H&E stained slice of aorta

Aorta, which stored in formalin solution, were made into the cross section and observed by the Olympus IX-71 camera (Olympus corporation, Tokyo). As shown in Figure 19, compared with control group, thickness of vessel wall slightly changed in SHXXTM-treated groups and the BB combination

group.

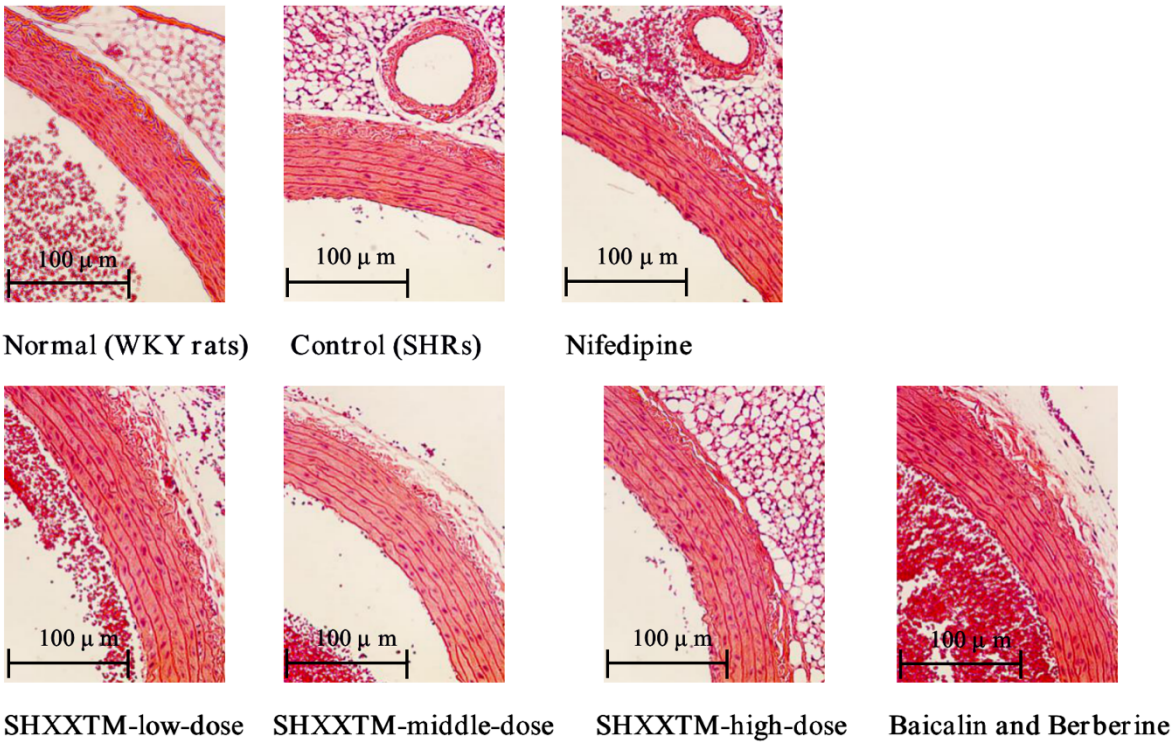


Figure 19 The photograph of H&E stained slice of aorta

2.4 Results of BB combination on NA-induced vascular contraction in endothelium-denuded strips and intact rings when pretreated with inhibitors and activators

To clarify which channels or pathways are involved in the vasorelaxant effects of SHXXTM, a preliminary *in vitro* experiments of BB combination with inhibitors and activators were proceeded (Table 15). The results are shown in Figures 20, 21, and 22. The results showed that the vasorelaxant effect was increased (10 μg/mL) in endothelium-intact rings compared to the endothelium-denuded strips, and after pretreatment with L-NAME in the rings, the vasorelaxant effect was obviously reduced (10 μg/mL). When pretreatment with rottlerin and calphostin C in endothelium-denuded strips, the vasorelaxant effect was increased (10 μg/mL), especially calphostin C, while pretreatment with a K_{ATP} channel activator (diazoxide, 1×10^{-4} M) slightly enhanced the vasorelaxant effects produced by the BB combination, whereas pretreatment with Kv blockers (4-AP, 1×10^{-4} M and TEA, 3×10^{-4} M), K^+ blocker (Ba^{2+}, 3×10^{-4} M), and K_{ATP} blocker (glibenclamide, 10^{-6} M) did not yield obvious changes of the vasorelaxant effects.

Table 15 Different inhibitors and activators

Name	Classification	Final Con. in medium (μM)
N^G-nitro-L-arginine methyl ester	NO blocker	100
Rottlerin	BKca activator	30
Calphostic C	PKC inhibitor	0.2
4-Aminopyridine (4-AP)	Kv blocker	100
Tetraethylammonium chloride (TEA)	Kv blocker	300
Barium chloride dihydrate (Ba^{2+})	K^+ blocker	300
Glibenclamide	K_{ATP} blocker	1
Diazoxide	K_{ATP} activator	100

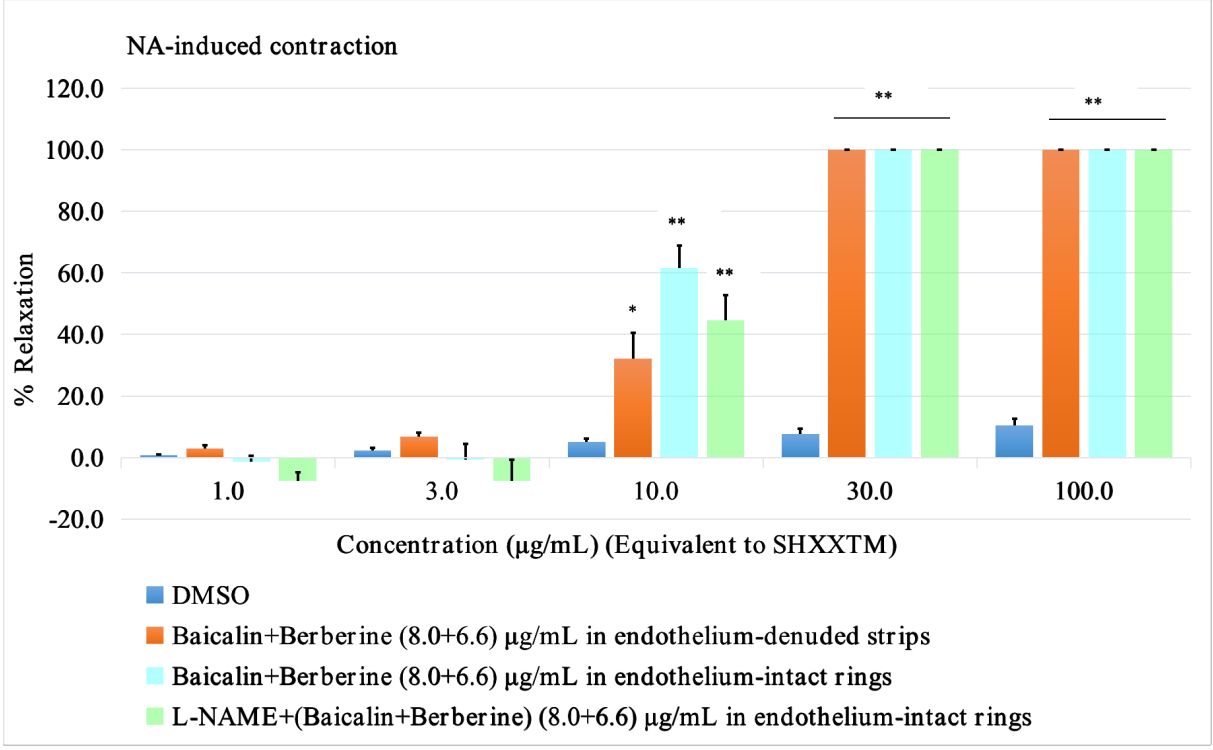


Figure 20 Effects of BB combination on NA-induced vascular contractions in endothelium-denude strips and endothelium-intact rings after pretreatment with L-NAME

Each bar graph represents the mean with S.E.M. (n = 4–6), *p<0.05 and **p<0.01 vs. DMSO control group.

[*J. Nat. Med.* (2020), Fig.6, https://doi.org/10.1007/s11418-019-01382-9]

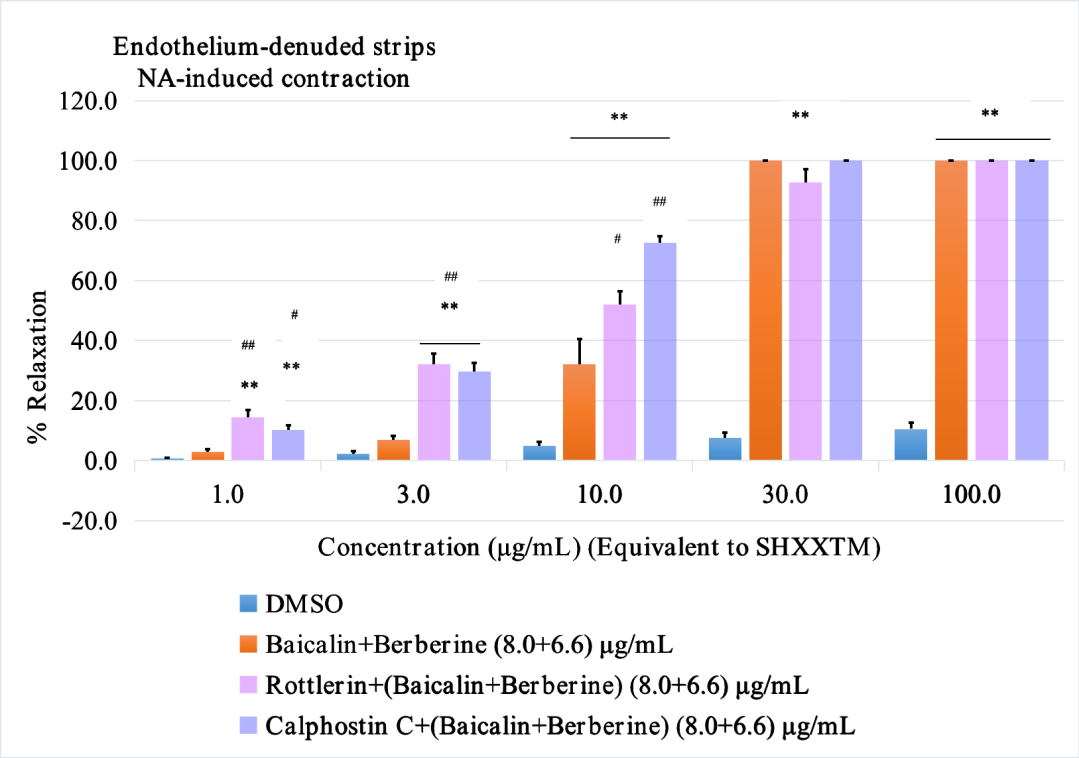


Figure 21 Effects of BB combination on NA-induced vascular contractions in endothelium-denude strips after pretreatment with rottlerin and calphostin C

Each bar graph represents the mean with S.E.M. (n = 4–6), *p<0.05 and **p<0.01 vs. DMSO control group, and $^{\#}p$<0.05 and $^{\#\#}p$<0.01 vs. the BB combination group.

[*J. Nat. Med.* (2020), Fig.6, https://doi.org/10.1007/s11418-019-01382-9]

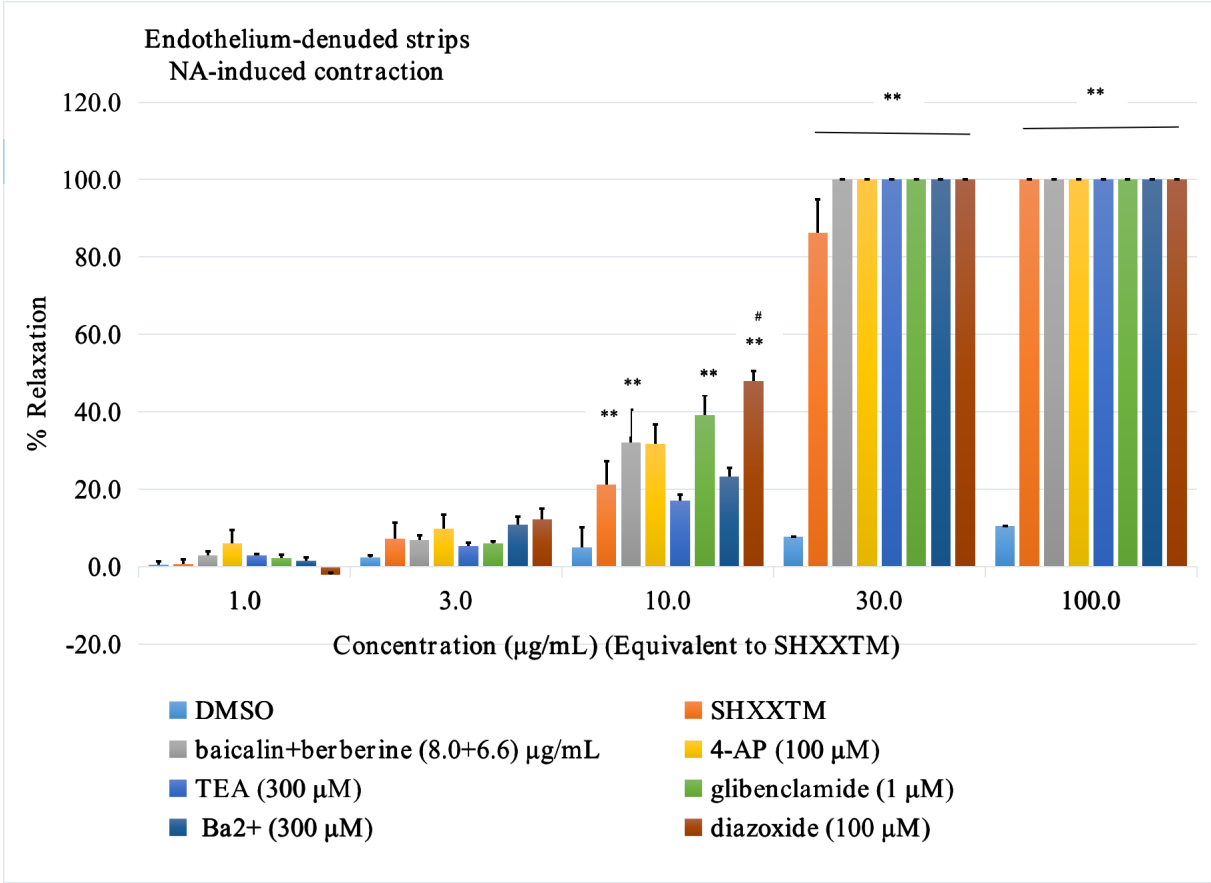


Figure 22 Effects of BB combination on NA-induced vascular contractions in endothelium-denude strips after pretreatment with 4-AP, TEA, glibenclamide, Ba^{2+}, and diazoxide

Each value represents the mean ± S.E.M. (n = 4–6), $^{**}p<0.01$ vs. DMSO control group, $^{\#}p<0.05$ vs. the BB combination group.

[*J. Nat. Med.* (2020), Fig.1, https://doi.org/10.1007/s11418-020-01387-9]

3. Discussion

From the results of *in vivo* study, from the fourth week onward, compared to the control group, the SHXXTM-low-dose group, the SHXXTM-middle-dose group, the SHXXTM-high-dose group, and the BB combination group significantly reduced increase in the rate of SBP increase. The results indicated that the SHXXTM groups and the BB combination exhibited a significant antihypertensive effects *in vivo*. This was consistent with the *in vitro* study, and it also suggested that BB combination might replace SHXXT in the clinical treatment in future. Meanwhile, as we described in the introduction, usually, the side effects of traditional medicines are less than synthetic drugs. But actually, in clinical, SHXXT was reported to cause interstitial pneumonia, hepatosis and jaundice [52]. In this study, maybe because of the short time for oral administration, the side effects on rats were not be observed. In future experiments, the side effects would be concerned and discussed .

It is well known that the left ventricle of heart is responsible for pumping blood into the arteries, the lungs, and the rest of the body. During hypertension, the left ventricle has to work harder to accomplish this process and becomes hypertrophied. In this study, in the SHXXT middle- and high-dose groups and the BB group, reduction of left ventricular hypertrophy was observed, and their values were almost the same as the nifedipine group. Hypertension is calculated by multiplying cardiac output (CO) and systemic vascular resistance (SVR) . SVR is usually depend on the vessel diameter, namely hypertension makes the vessel wall thickened, then the SVR is increased and causes hypertension. In this study, the SHXXTM and the BB combination group exhibited a tendency to reduce the thickness of the vessels with the long-term oral administration.

To understand the vasorelaxant effects mechanism of BB combination, some inhibitors or activators were used in the *in vitro* antivascular experiments to make a preliminary assessment. From the results, it was speculated that the endothelium was involved in the vasorelaxant effects. Nitric oxide (NO), which is released by endothelial cells, diffuses into vascular smooth muscle cells where it will activate soluble guanylate cyclase (sGC), which in turn catalyzes the production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP), then, cGMP induces vascular smooth muscle relaxation by activating cGMP-dependent protein kinase G (PKG) [53]. Liu *et al*. showed that cGMP/PKG phosphorylates BK_{Ca} subunit to activate the BK_{Ca} channel, which induces vascular smooth muscle membrane hyperpolarization and subsequently causes vasorelaxation [54]. Therefore, it was speculated that BB combination would promote NO release from the endothelium to activate the NO/cGMP pathway, thereby inducing vasorelaxation. On the other hand, PKG was reported to directly decrease intracellular Ca^{2+} increase, which contributed to vascular contraction [55], although more evidence is needed to verify this. With regard to the BK_{Ca} channel, studies have suggested that high blood pressure and vascular dysfunction are involved in cellular signaling cascades that alter arterial BK_{Ca} channel expression to modify vascular tone further [56, 57]. In this study, we also used rottlerin, a

BK_{Ca} channel opener [58], for vessel pretreatment in the *in vitro* experiment, and the results showed that the relaxation effect was increased. This result suggested that the BK_{Ca} channel might be involved in the vasorelaxant effects of SHXXTM, consistent with previous findings [22, 25]. Meanwhile, it was found that after pretreatment with calphostin C, a protein kinase C (PKC) inhibitor, the vasorelaxant effects was also increased, and the enhancement was greater than that by rottlerin. This result indicated that the DAG/PKC/CPI-17 pathway might be involved in the vasorelaxant effects. C-kinase potentiated protein phosphatase-1 inhibitor of 17 kDa (CPI-17), which dephosphorylates myosin light chain phosphatase (MLCP) into MLC_{20}, causes vasorelaxation. Recently, several studies have demonstrated that CPI-17 plays important roles in vascular smooth muscle function [59-62]. Beside these results, moderate effects on high K^+-induced contractions were observed, which suggesting that the Ca^{2+} channel was involved . The signaling pathways for vascular contraction are shown in Figure 23 [63, 64].

As described above, these results showed that the BB combination produced vasorelaxant effects by activating the NO/cGMP pathway in the endothelium-intact rings, and the VDCC channel, the BK_{Ca} channel, K_{ATP} channel, and the DAG/PKC/CPI-17 pathway might be involved. But here, actually, these results are only a hypothesis or speculation, because only the *in vitro* experiments are not enough to make the decision, for example, rottlerin or calphostin C might have some effects on the vascular which would cause the relaxion or contraction [65, 66], so more experiments such as westernblot analysis of signal proteins or Ca^{2+} detection are needed to verify.

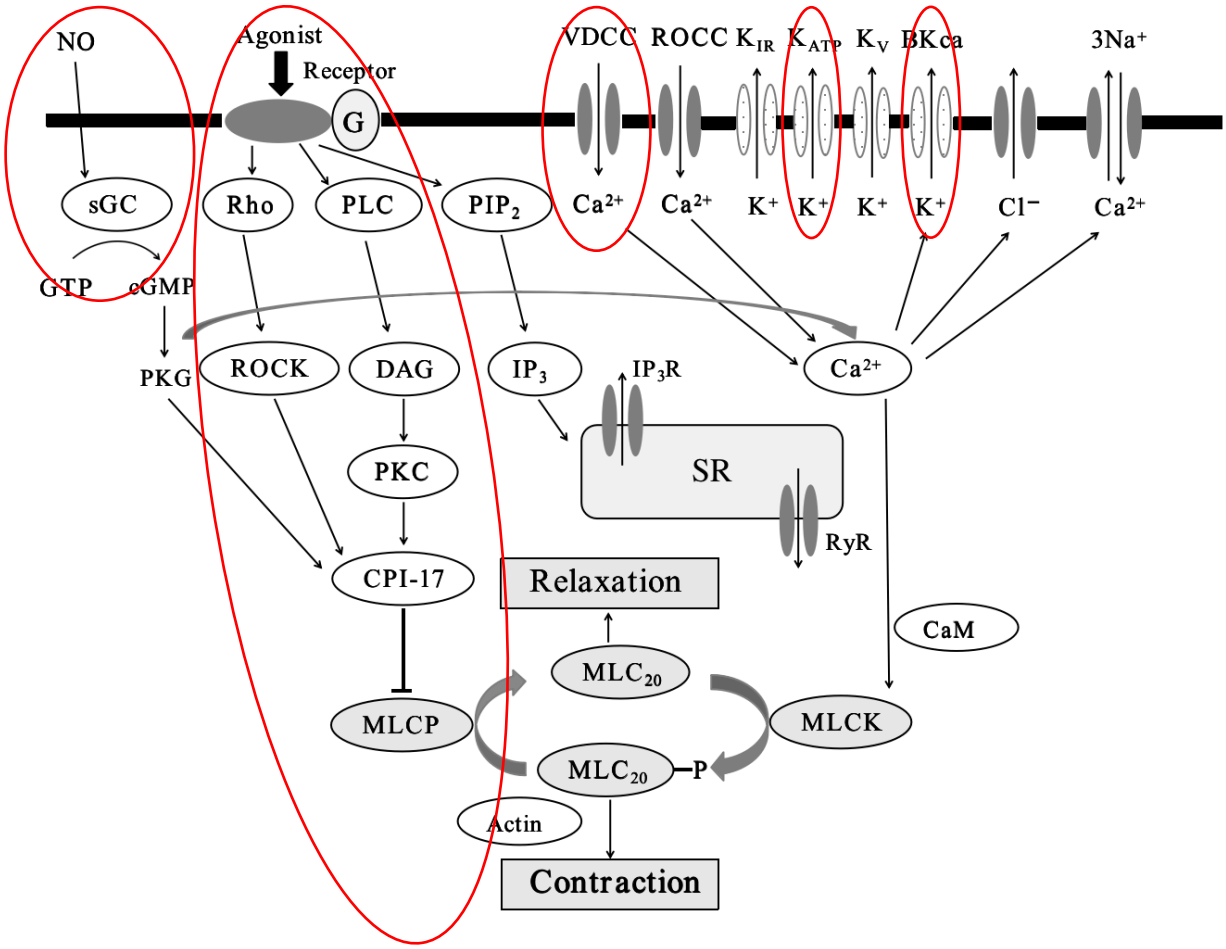


Figure 23 Signaling pathway for vascular smooth muscle contraction

VDCC: voltage-dependent calcium channel; ROCC: receptor-operated calcium channel; KIR: inward rectifier potassium channel; KATP: ATP-sensitive potassium channel; KV: voltage-gated K^+ channel; BKCa: Ca^{2+}-activated K^+ channel; sGC: soluble guanylyl cyclase; Rho: Rho-associated kinase; PLC: phospholipase C; PIP2: phosphatidylinositol (4,5)-bisphosphate; GTP: guanosine triphosphate; cGMP: cyclic guanosine monophosphate; PKG: protein kinase G; ROCK: Rho-associated protein kinase; DAG: diacylglycerol; IP3: inositol trisphosphate; SR: sarcoplasmic reticulum; PKC: protein kinase C; RyR: ryanodine receptor; CPI-17: C-kinase potentiated protein phosphatase-1 inhibitor of 17 kDa; CaM: calmodulin; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase; MLC20: 20 kDa myosin light chain.

Conclusion

In this study, from all the results, the conclusions were as follows:

1. PCA and PCR are suitable and reliable methods for scientific research of multicomponent analysis, especially for traditional Chinese medicines, Kampo medicines and some folk medicines. Connected with pharmacological data, it could simplify the complex constituents of medicines.

2. In this study, it showed that Sanoshashinto had significant vasorelaxant effects *in vitro* and antihypertensive effects *in vivo*. Baicalin and berberine, which were the main antihypertensive constituents of Sanoshashinto, in future, it might replace Sanoshashinto for using as an antihypertensive drug in the clinical.

3. This study also speculated that the baicalin and berberine combination produced vasorelaxant effects by activating the NO/cGMP pathway in the endothelium-intact rings. Beside this, the preliminary study showed that VDCC channel were also involved. About the other channels and pathways, more experiments are needed to verify.

Acknowledgement

First, I would like to express my best and sincere thanks to my supervisor, professor Hisashi Matsuda, for providing me the precious opportunity as a Ph.D. student in his laboratory. In the four years, I learned a lot of from Professor Matsuda, not only the experiments, but also the attitude of diligent, earnest, assiduous, and Professor Matsuda will be always the role model for me.

I would like to give my sincere thanks to associate professor Seikou Nakamura, kindly assistant professor Souichi Nakashima, they gave lots of suggestions and help to me, which made me to adapt the study and the life of Japan.

I also greatly appreciate professor Satoshi Tanaka and professor Yoshitaka Yano, from them, I got a lot of nice suggestions and advices for my doctor research and thesis.

I also would like to give my special thanks to professor Tatsuya Kitade, professor Tetsuo Nakata, associate professor Miyuki Kobara, associate professor Shigehiko Takegami, assistant professor Atsuko Konishi. They helped me a lot in the experiments, especially associate professor Shigehiko Takegami, he teached me lots of acknowledges of analysis.

I would like to thank professor Kenichi Inui and professor Changqing Yang for their guidance in my academic study.

Also I would like to thank Dr. Keiko Ogawa, Dr. Masashi Fukaya, Dr. Yoshimi Oda, Dr. Weicheng Wang, Ms. Mamiko Yano, Ms. Tomoko Matsumoto, Ms. Kaori Ryu, Mr. Taichi Yoneda, Mr. Takaya Iwamoto, Ms. Ayako Ohta, Ms. Nami Momura, Ms. Marina Shigyo, Ms. Mutsumi Yamasaki, Ms. Sumire Ikuno, Ms. Aoi Morikawa, both of them helped me a lot in experiments and life, we got along very well with each other, and when talking with them, my Japanese language got the good progress.

In particular, my dear wife, Ms. QianZhou, whose gorgeous smile encouraged me to be more than I can be, her support and understanding is my power to create a better future.

Finally, I am grateful to everyone in my life. Many thanks for everyone's insightful comments and generous help.

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Supplementary Materials

Figures:

Figure S1 The vasorelaxant effects graphs of all samples and fractions on NA-induced contractions in endothelium-denuded strips

Figure S2 HPLC data of all samples and fractions

Figure S3 The PCA original data

Figure S4 The results of PCA analysis

Figure S5 The results of PCR analysis

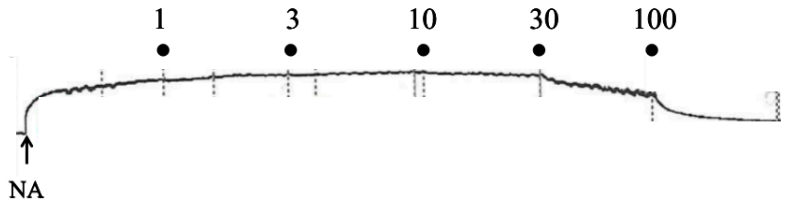

Figure S1.1 Effects of SHXXTM-W on NA-induced contractions in endothelium-denuded strips

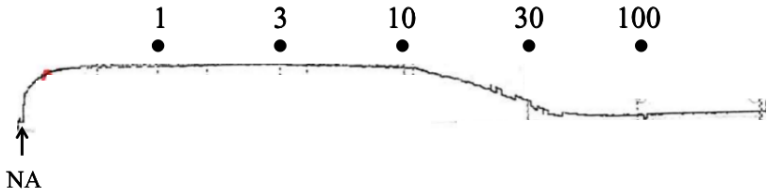

Figure S1.2 Effects of SHXXTM-Bu on NA-induced contractions in endothelium-denuded strips

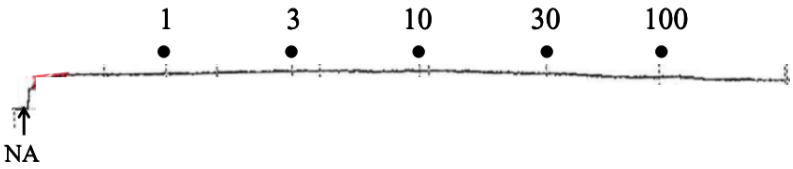

Figure S1.3 Effects of SHXXTM-EA on NA-induced contractions in endothelium-denuded strips

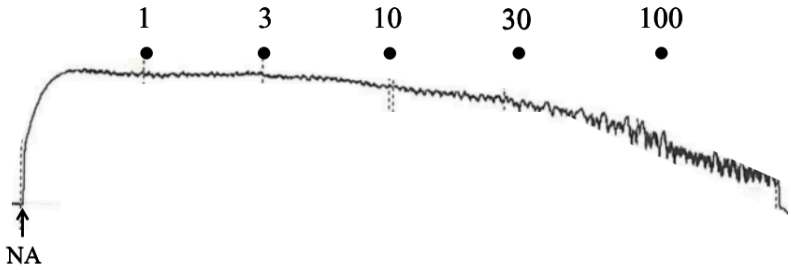

Figure S1.4 Effects of DHM on NA-induced contractions in endothelium-denuded strips

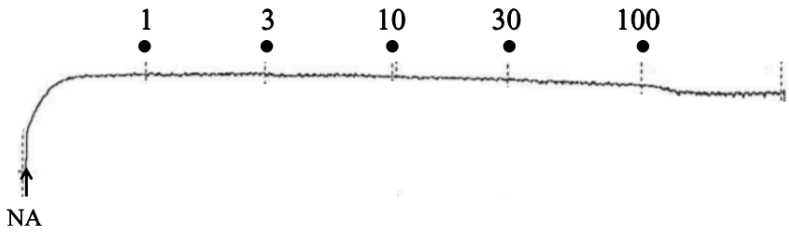

Figure S1.5 Effects of DHM-W on NA-induced contractions in endothelium-denuded strips

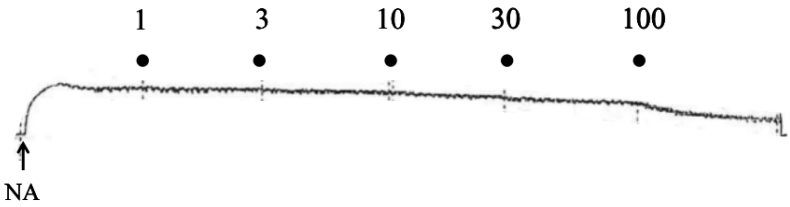

Figure S1.6 Effects of DHM-Bu on NA-induced contractions in endothelium-denuded strips

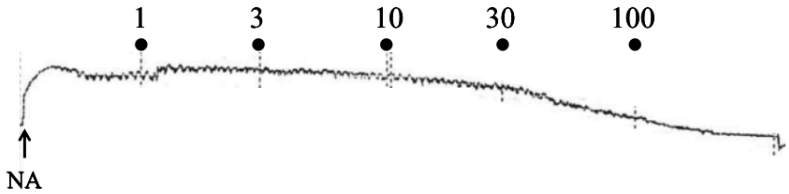

Figure S1.7 Effects of DHM-EA on NA-induced contractions in endothelium-denuded strips

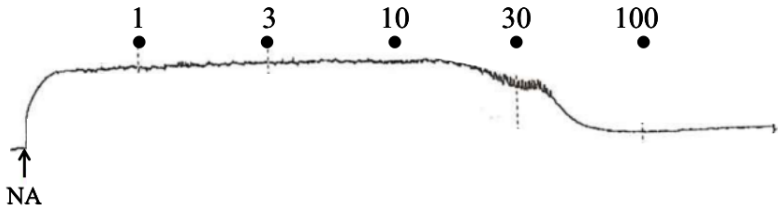

Figure S1.8 Effects of HQM on NA-induced contractions in endothelium-denuded strips

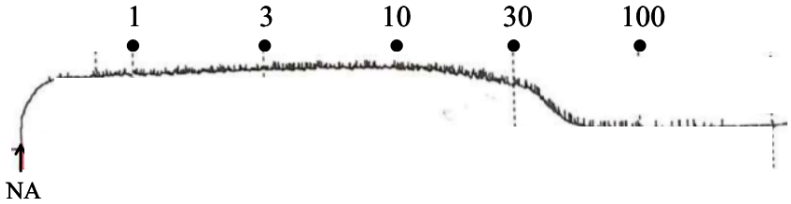

Figure S1.9 Effects of HQM-W on NA-induced contractions in endothelium-denuded strips

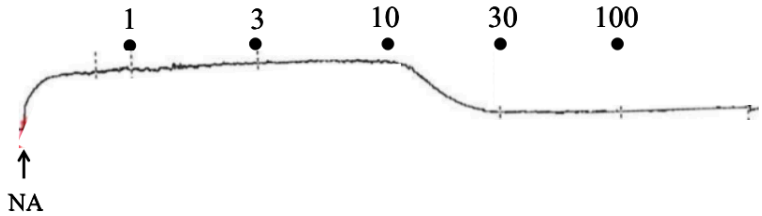

Figure S1.10 Effects of HQM-Bu on NA-induced contractions in endothelium-denuded strips

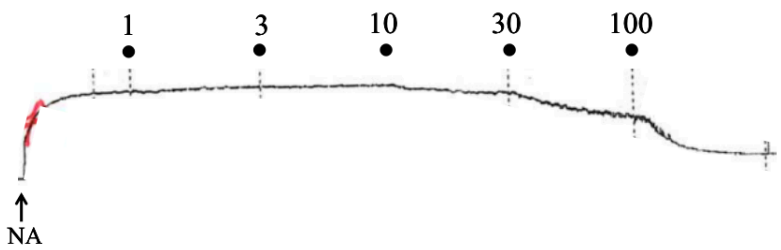

Figure S1.11 Effects of HQM-EA on NA-induced contractions in endothelium-denuded strips

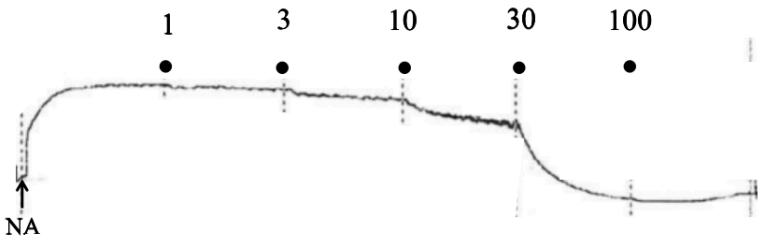

Figure S1.12 Effects of CHLM on NA-induced contractions in endothelium-denuded strips

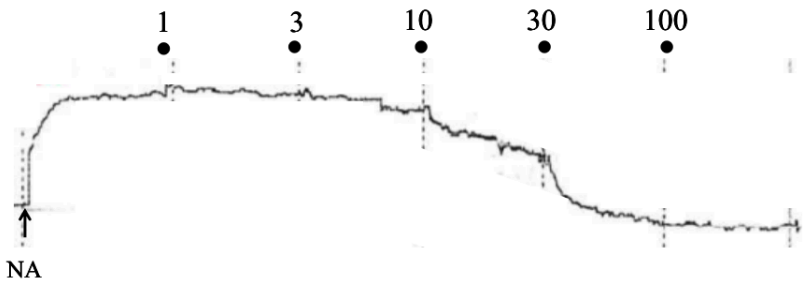

Figure S1.13 Effects of CHLM-W on NA-induced contractions in endothelium-denuded strips

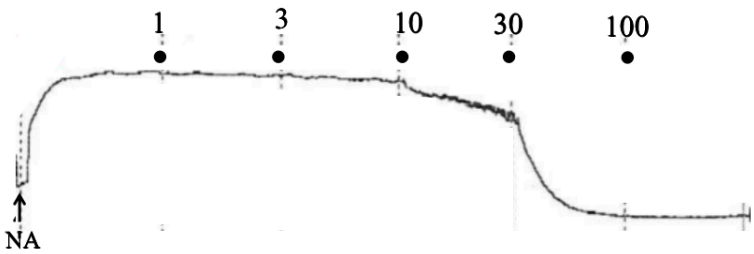

Figure S1.14 Effects of CHLM-Bu on NA-induced contractions in endothelium-denuded strips

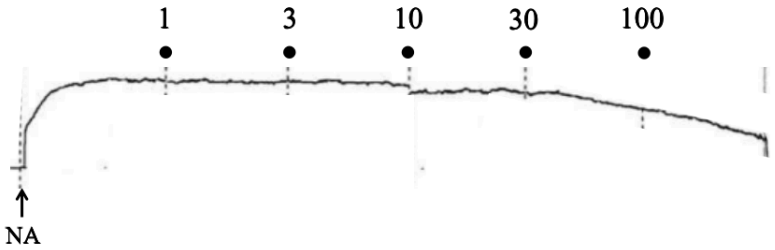

Figure S1.15 Effects of CHLM-EA on NA-induced contractions in endothelium-denuded strips

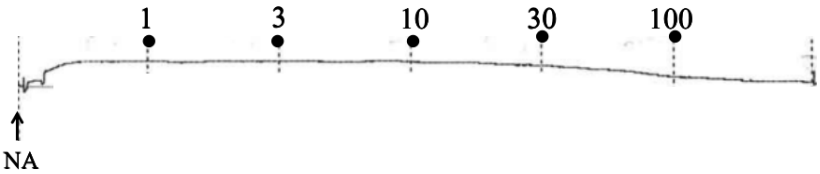

Figure S1.16 Effects of DHHQM on NA-induced contractions in endothelium-denuded strips

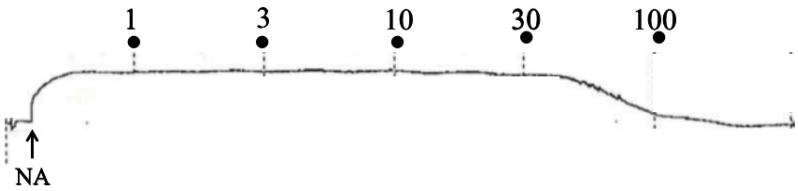

Figure S1.17 Effects of DHHQM-W on NA-induced contractions in endothelium-denuded strips

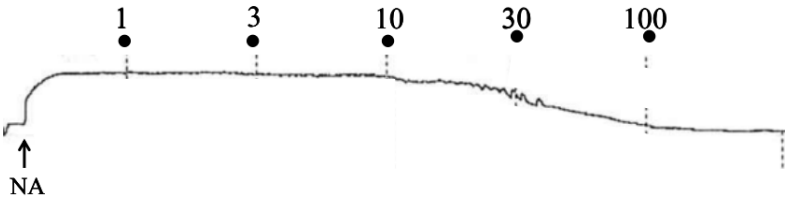

Figure S1.18 Effects of DHHQM-Bu on NA-induced contractions in endothelium-denuded strips

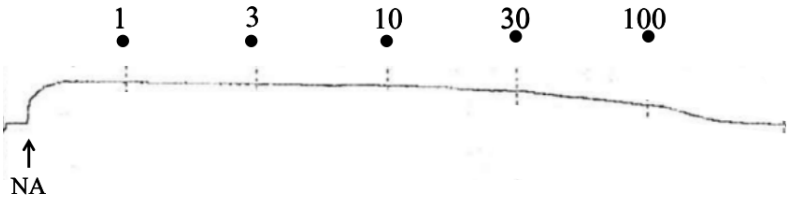

Figure S1.19 Effects of DHHQM-EA on NA-induced contractions in endothelium-denuded strips

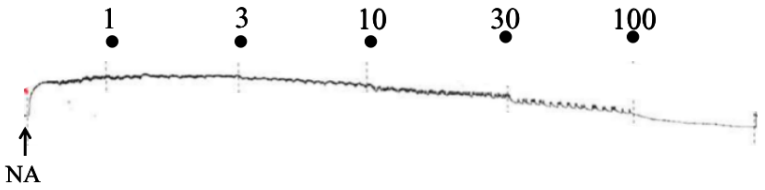

Figure S1.20 Effects of DHHLM on NA-induced contractions in endothelium-denuded strips

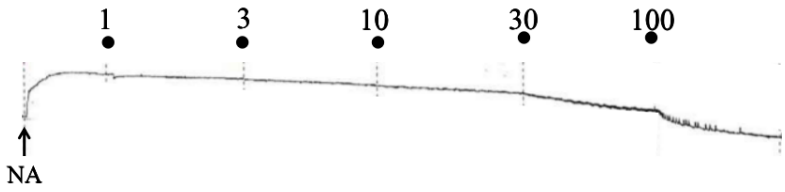

Figure S1.21 Effects of DHHLM-W on NA-induced contractions in endothelium-denuded strips

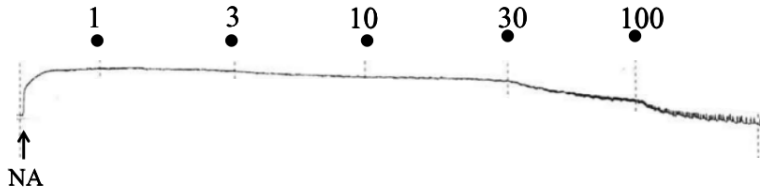

Figure S1.22 Effects of DHHLM-Bu on NA-induced contractions in endothelium-denuded strips

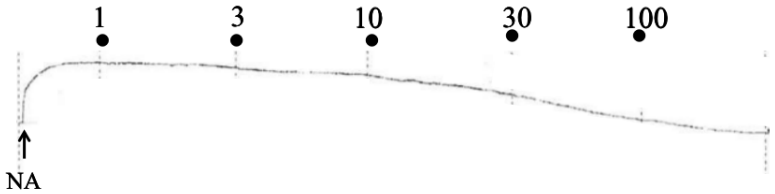

Figure S1.23 Effects of DHHLM-EA on NA-induced contractions in endothelium-denuded strips

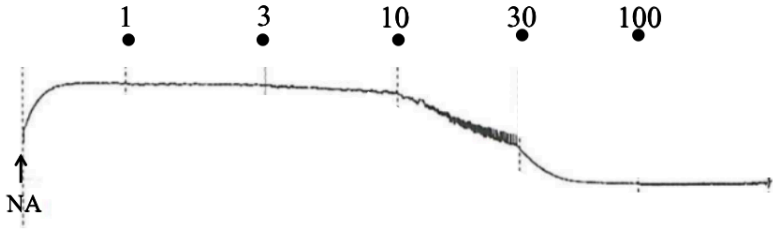

Figure S1.24 Effects of HQHLM on NA-induced contractions in endothelium-denuded strips

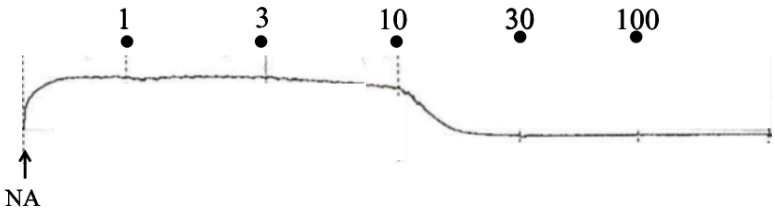

Figure S1.25 Effects of HQHLM-W on NA-induced contractions in endothelium-denuded strips

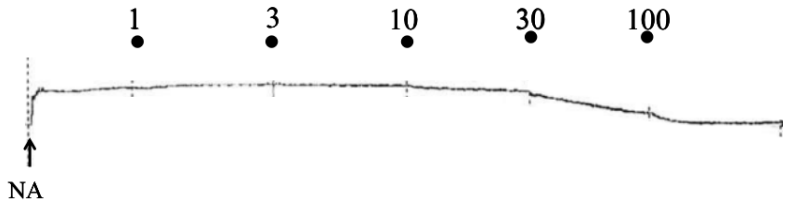

Figure S1.26 Effects of HQHLM-Bu on NA-induced contractions in endothelium-denuded strips

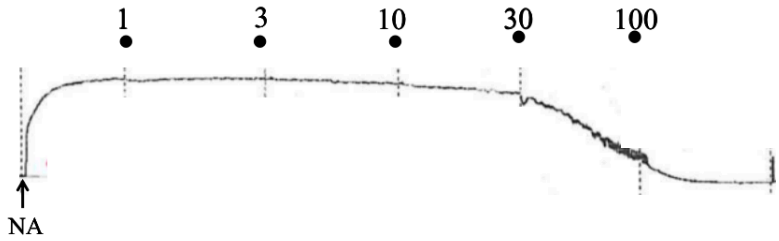

Figure S1.27 Effects of HQHLM-EA on NA-induced contractions in endothelium-denuded strips

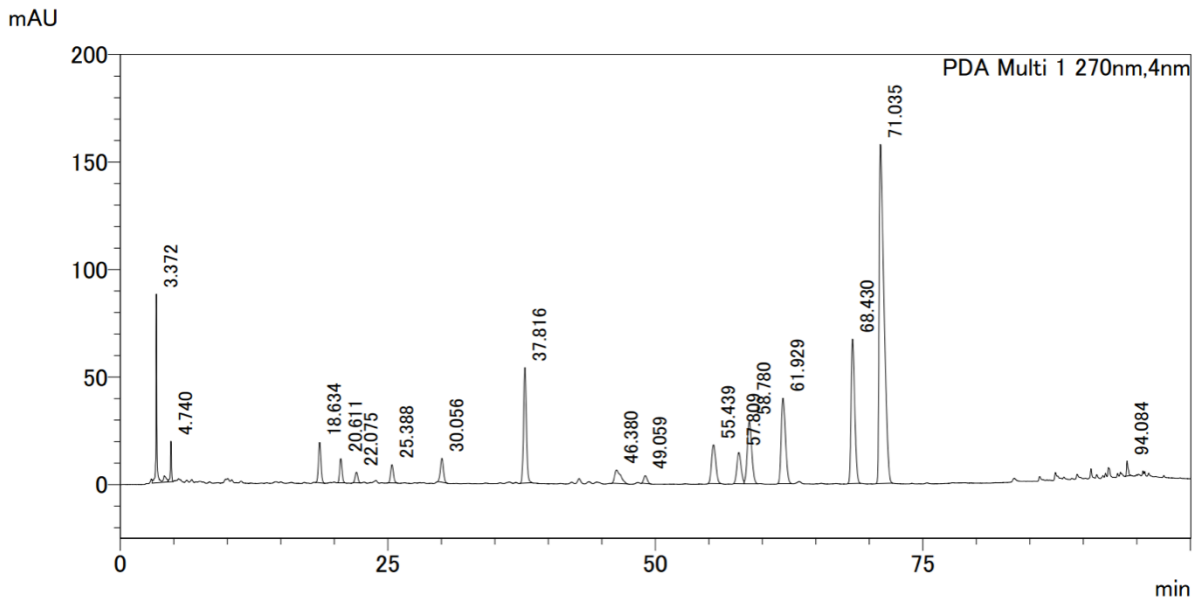

Figure S2.1 HPLC chromatogram of the water fraction of SHXXTM (SHXXTM-W)

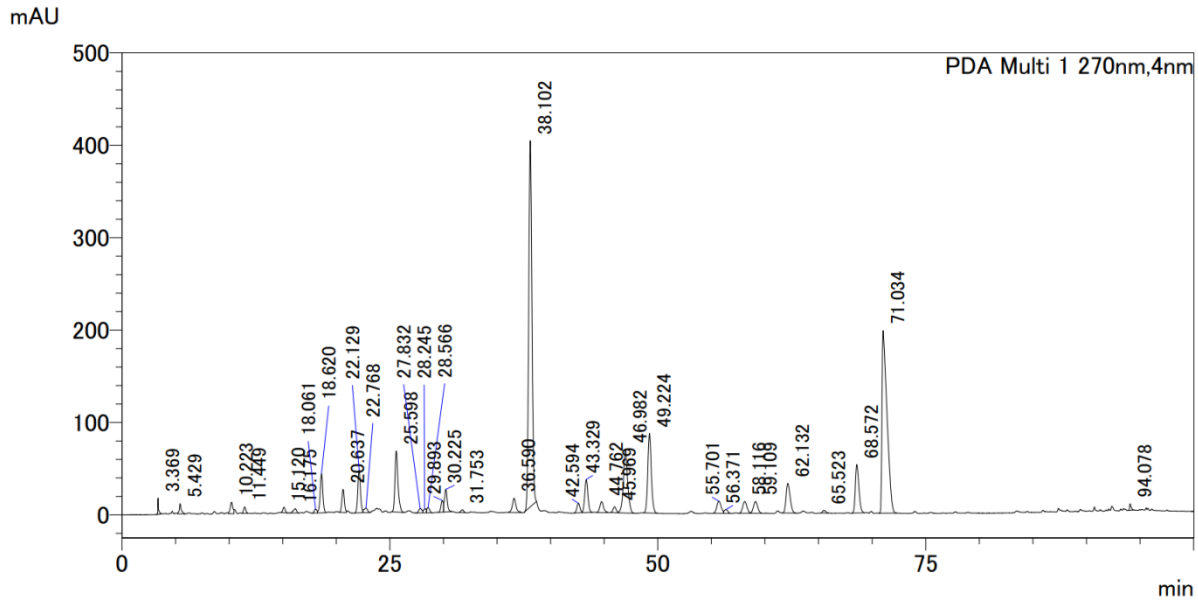

Figure S2.2 HPLC chromatogram of the *n*-butanol fraction of SHXXTM (SHXXTM-Bu)

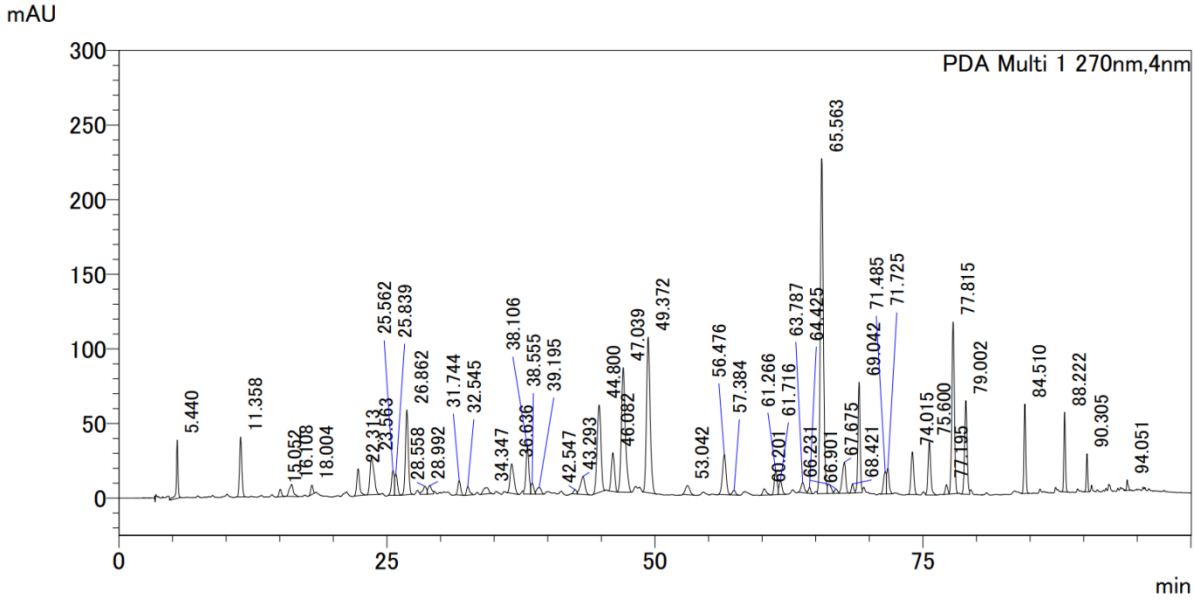

Figure S2.3 HPLC chromatogram of the ethyl acetate fraction of SHXXTM (SHXXTM-EA)

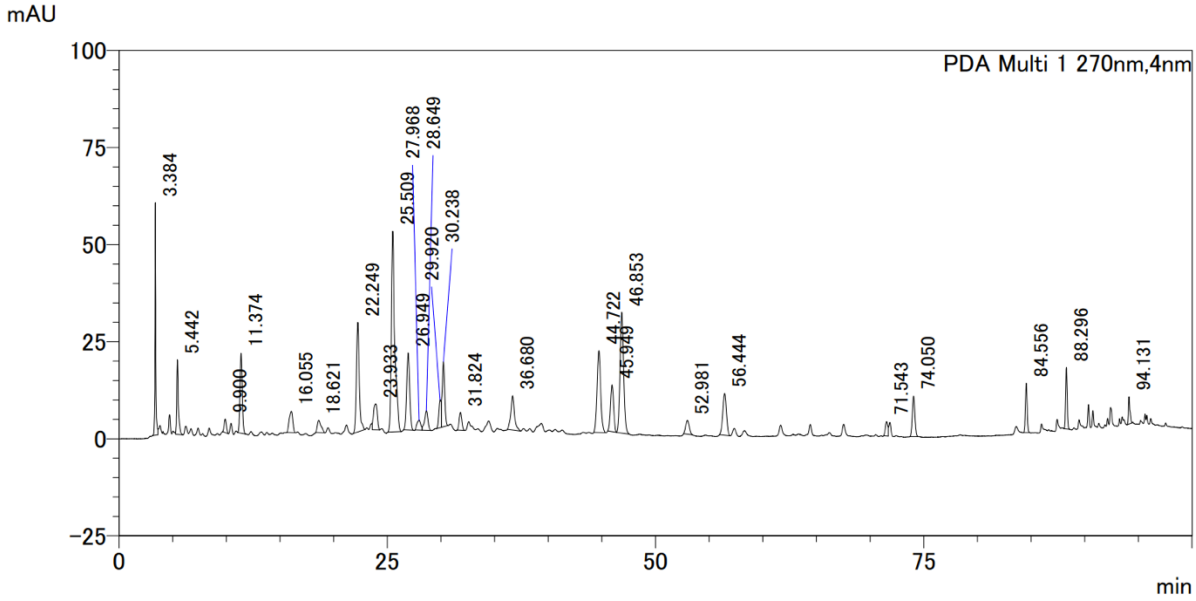

Figure S2.4 HPLC chromatogram of the methanol extract of DH (DHM)

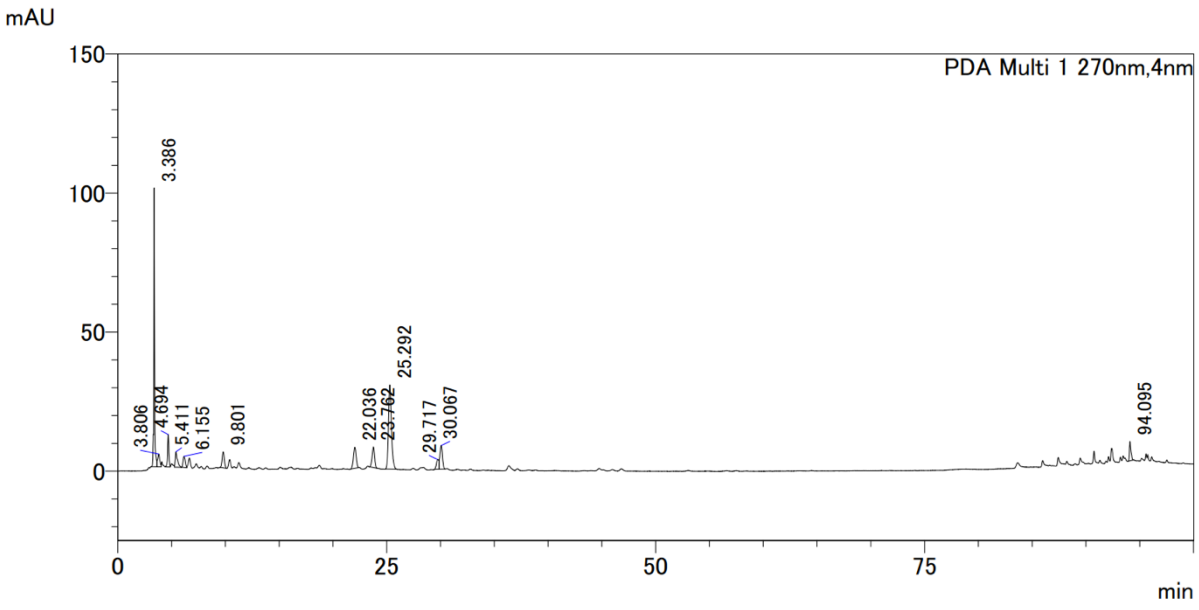

Figure S2.5 HPLC chromatogram of the water fraction of DH methanol extract (DHM-W)

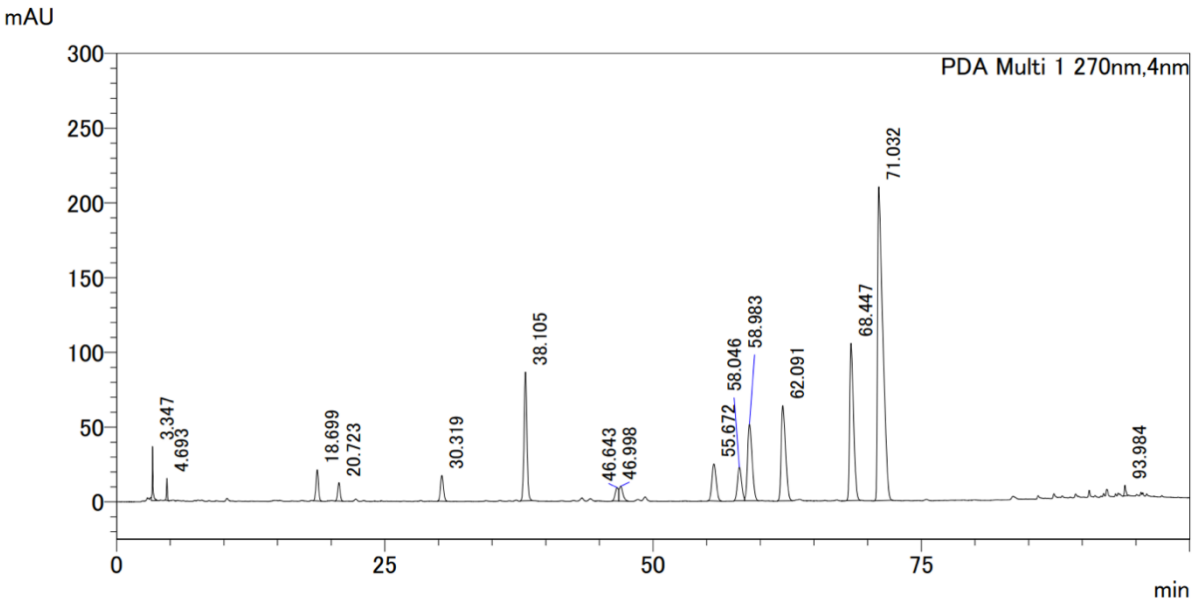

Figure S2.6 HPLC chromatogram of the *n*-butanol fraction of DH methanol extract (DHM-Bu)

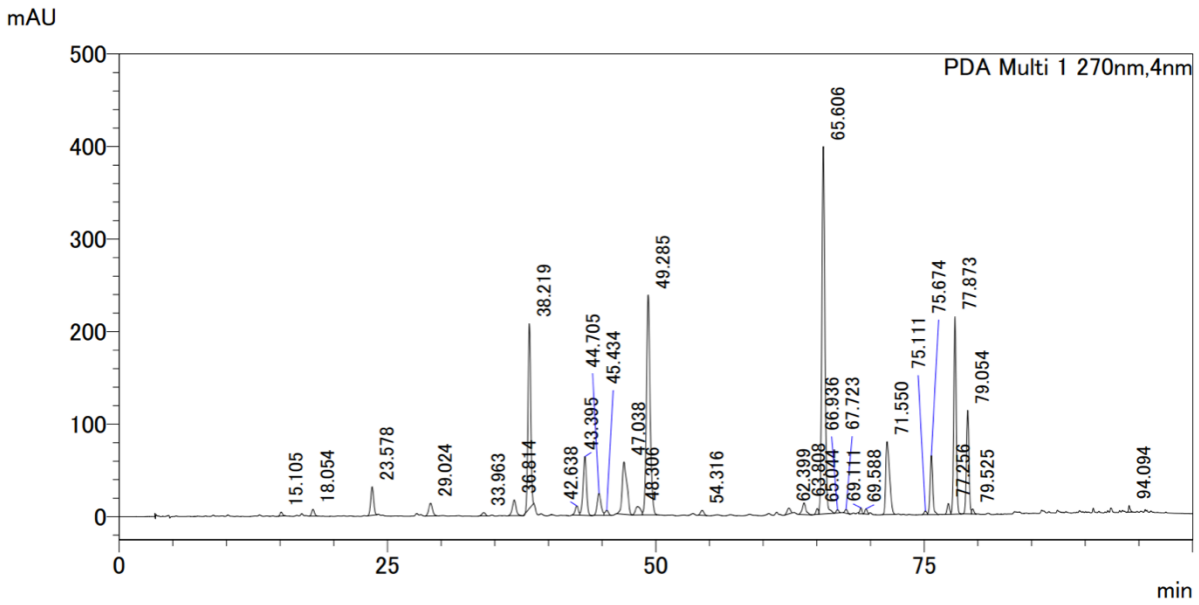

Figure S2.7 HPLC chromatogram of the ethyl acetate fraction of DH methanol extract (DHM-EA)

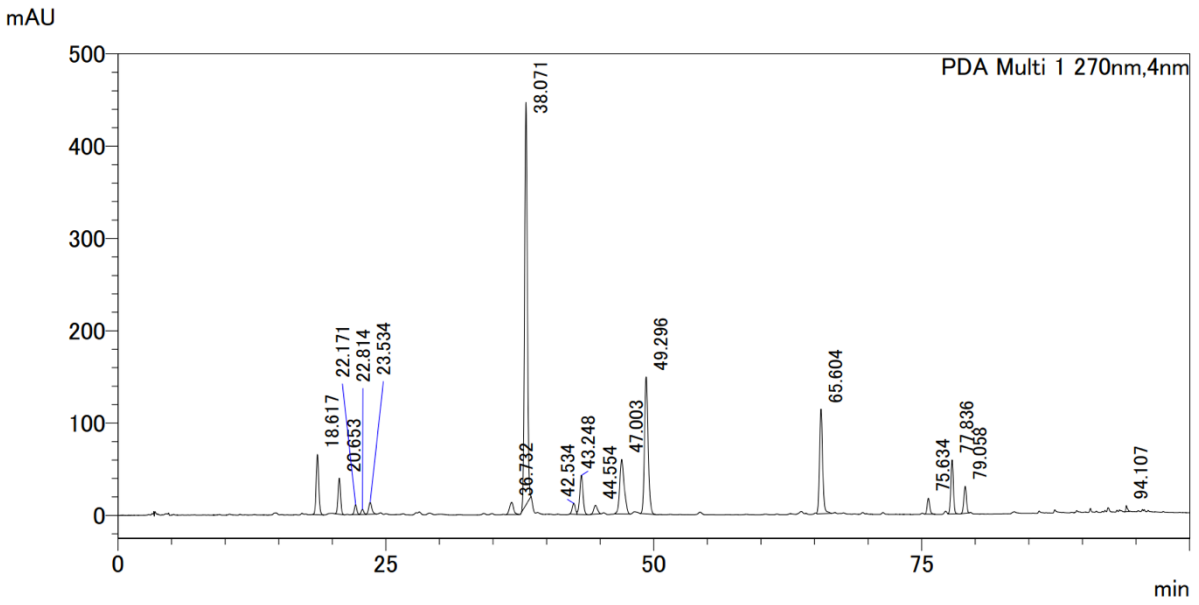

Figure S2.8 HPLC chromatogram of the methanol extract of HQ (HQM)

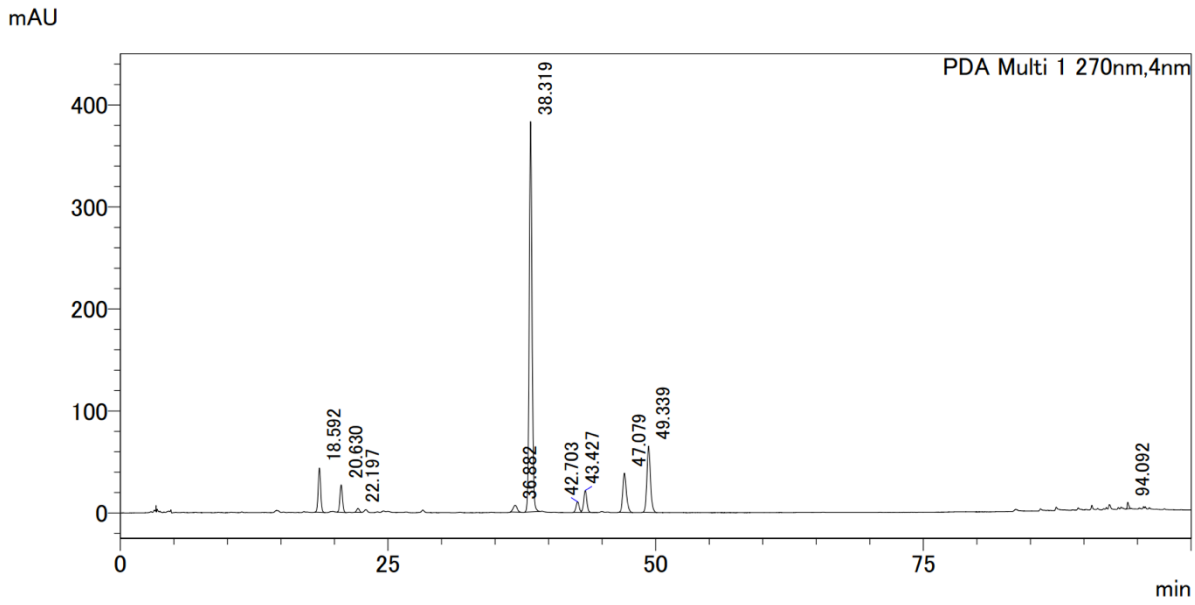

Figure S2.9 HPLC chromatogram of the water fraction of HQ methanol extract (HQM-W)

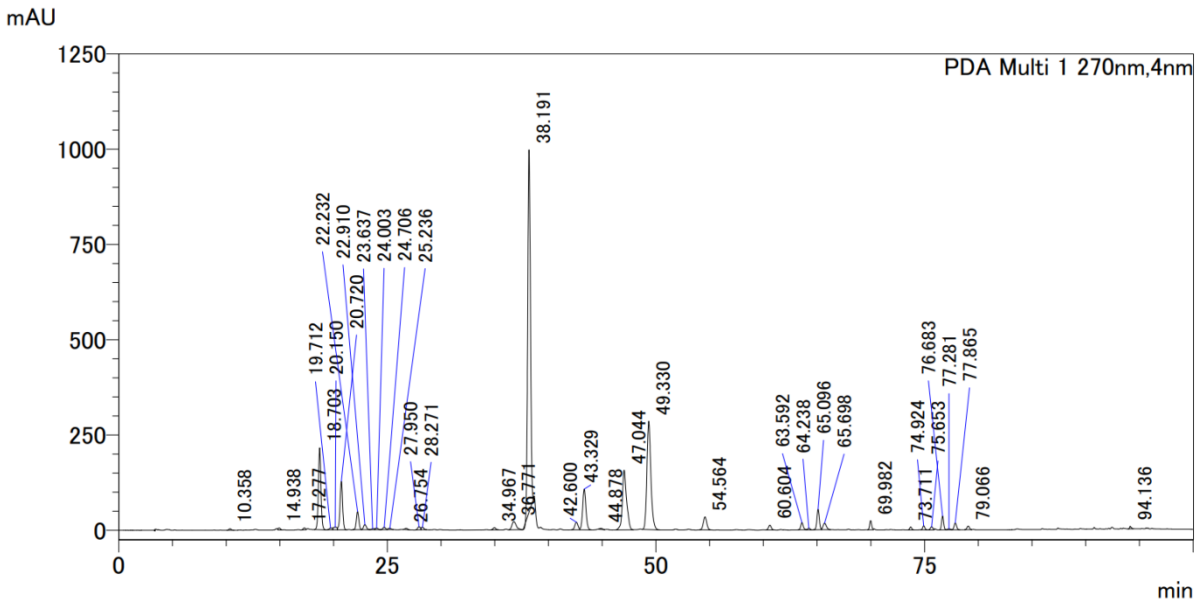

Figure S2.10 HPLC chromatogram of the *n*-butanol fraction of HQ methanol extract (HQM-Bu)

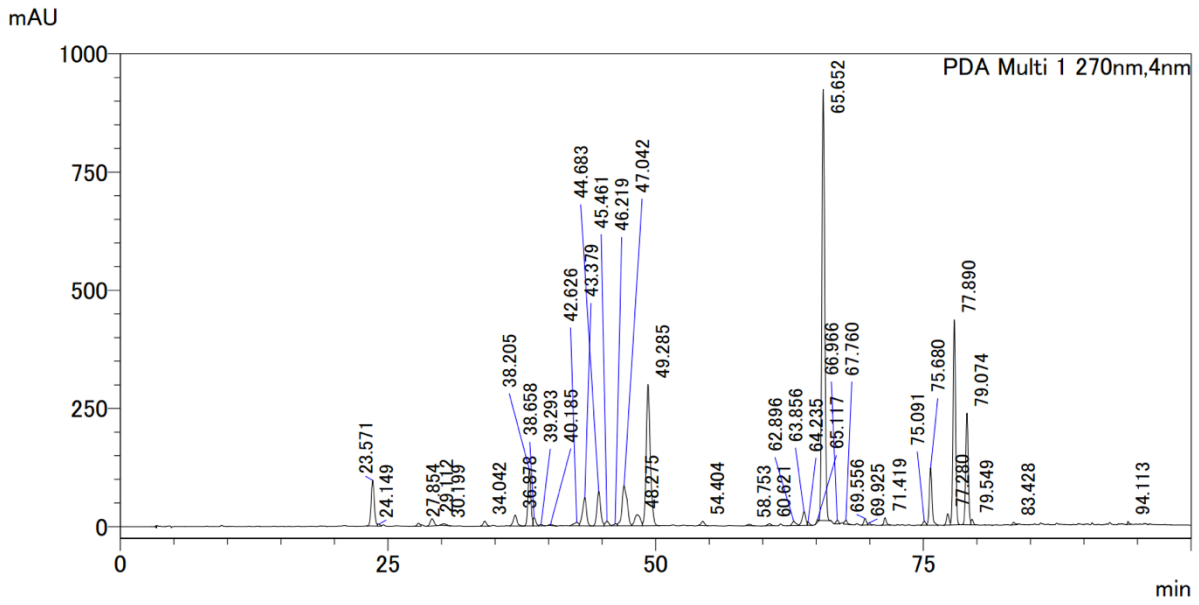

Figure S2.11 HPLC chromatogram of the ethyl acetate fraction of HQ methanol extract (HQM-EA)

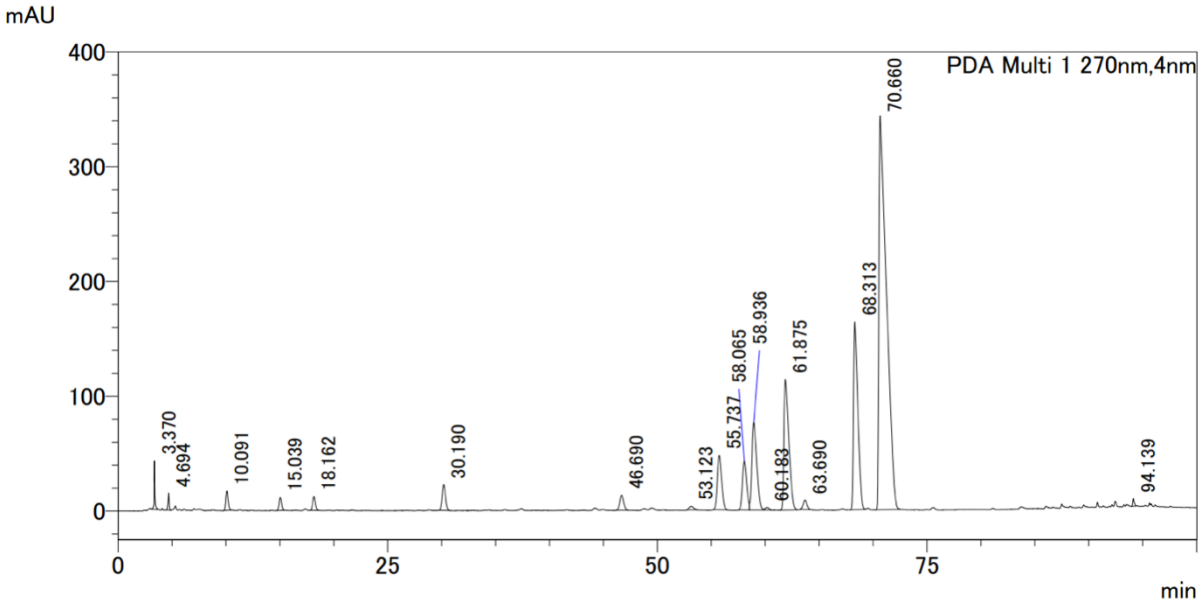

Figure S2.12 HPLC chromatogram of the methanol extract of CHL (CHLM)

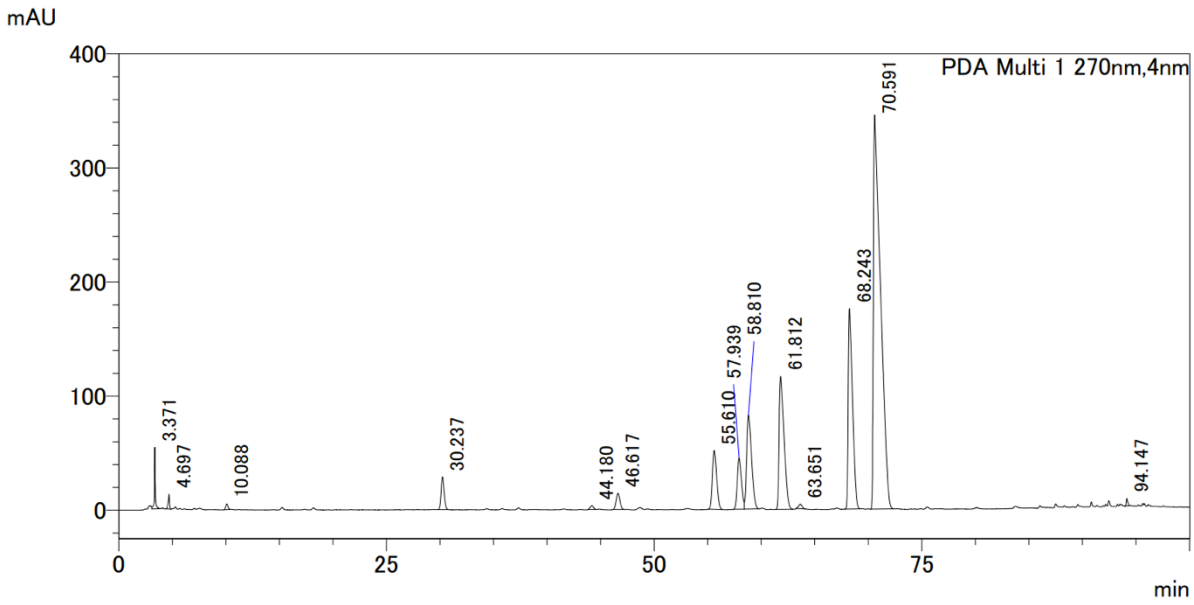

Figure S2.13 HPLC chromatogram of the water fraction of CHL methanol extract (CHLM-W)

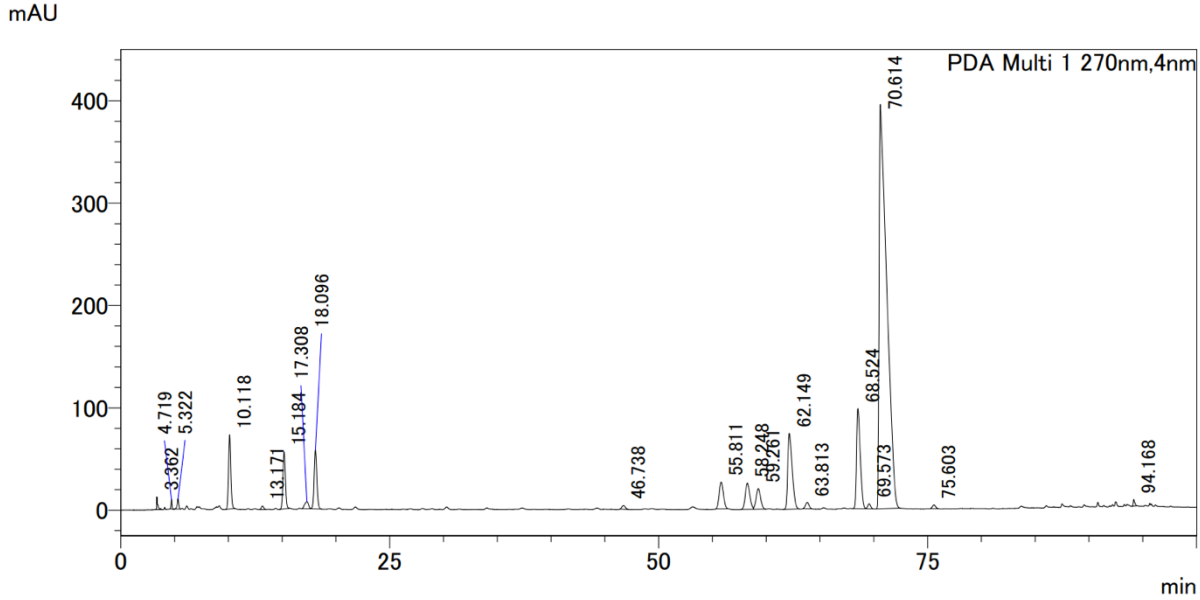

Figure S2.14 HPLC chromatogram of the *n*-butanol fraction of CHL methanol extract (CHLM-Bu)

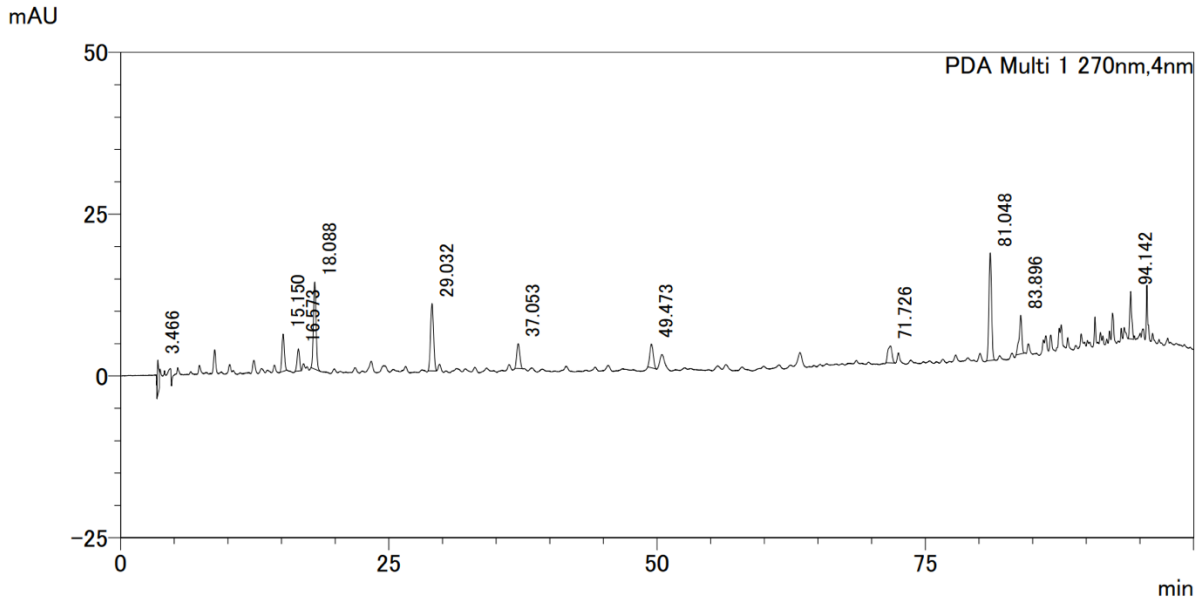

Figure S2.15 HPLC chromatogram of the ethyl acetate fraction of CHL methanol extract (CHLM-EA)

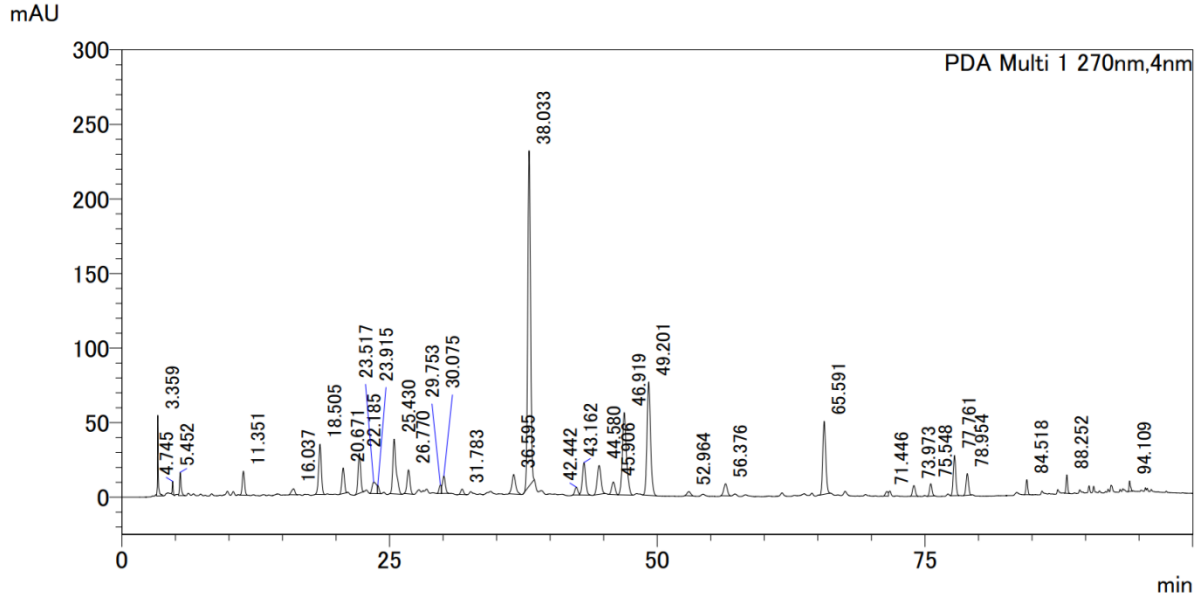

Figure S2.16 HPLC chromatogram of the methanol extract of DH and HQ (DHHQM)

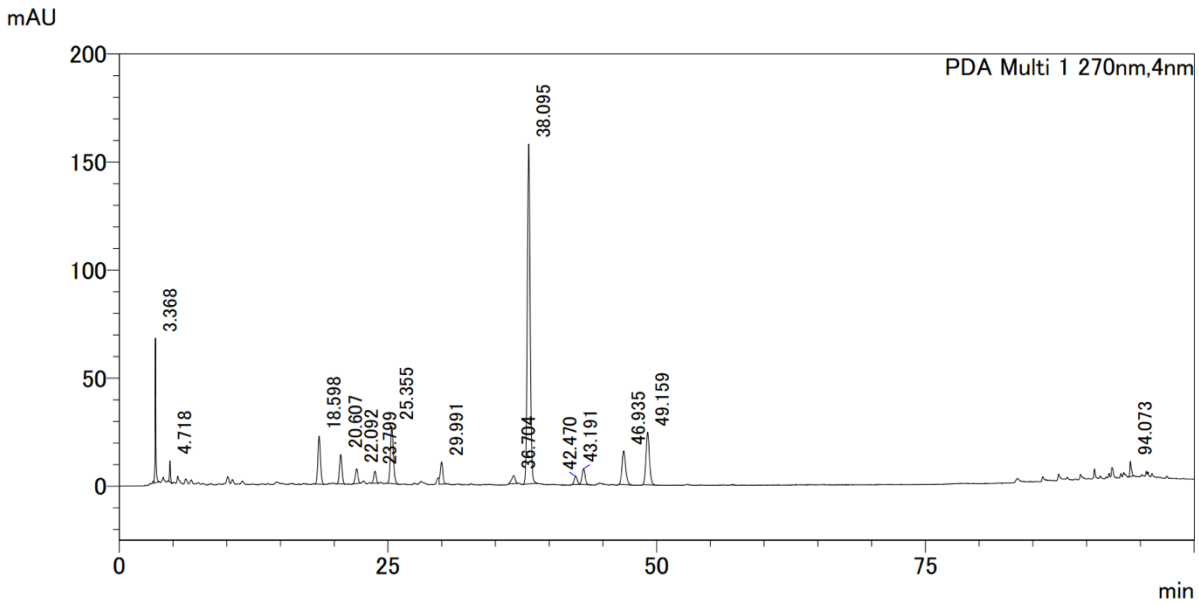

Figure S2.17 HPLC chromatogram of the water fraction of DH and HQ methanol extract (DHHQM-W)

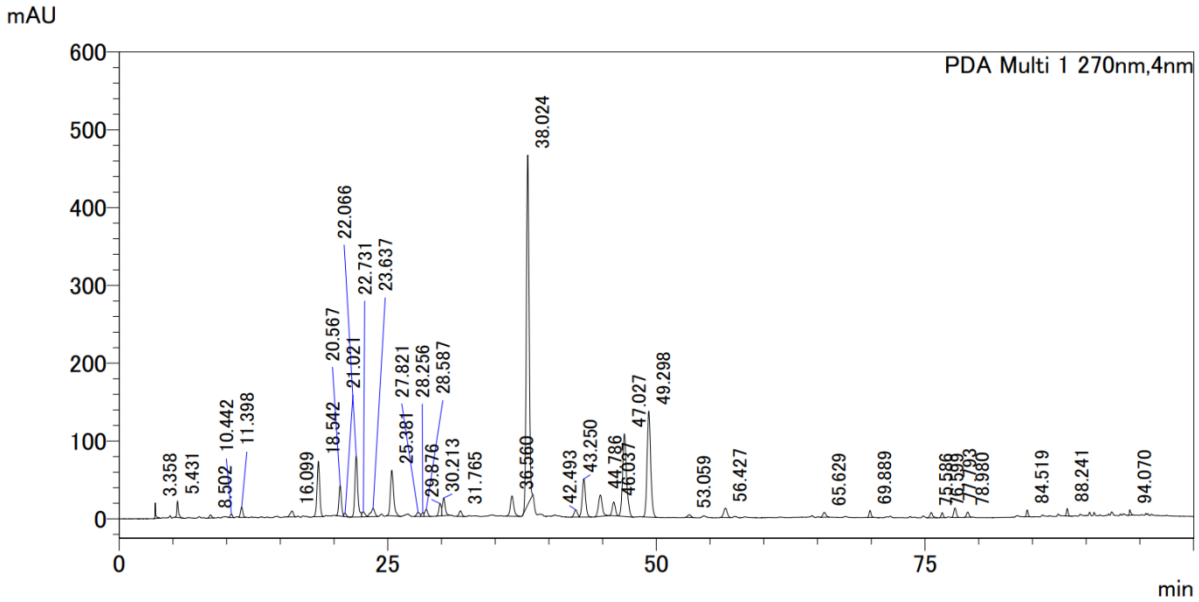

Figure S2.18 HPLC chromatogram of the *n*-butanol fraction of DH and HQ methanol extract (DHHQM-Bu)

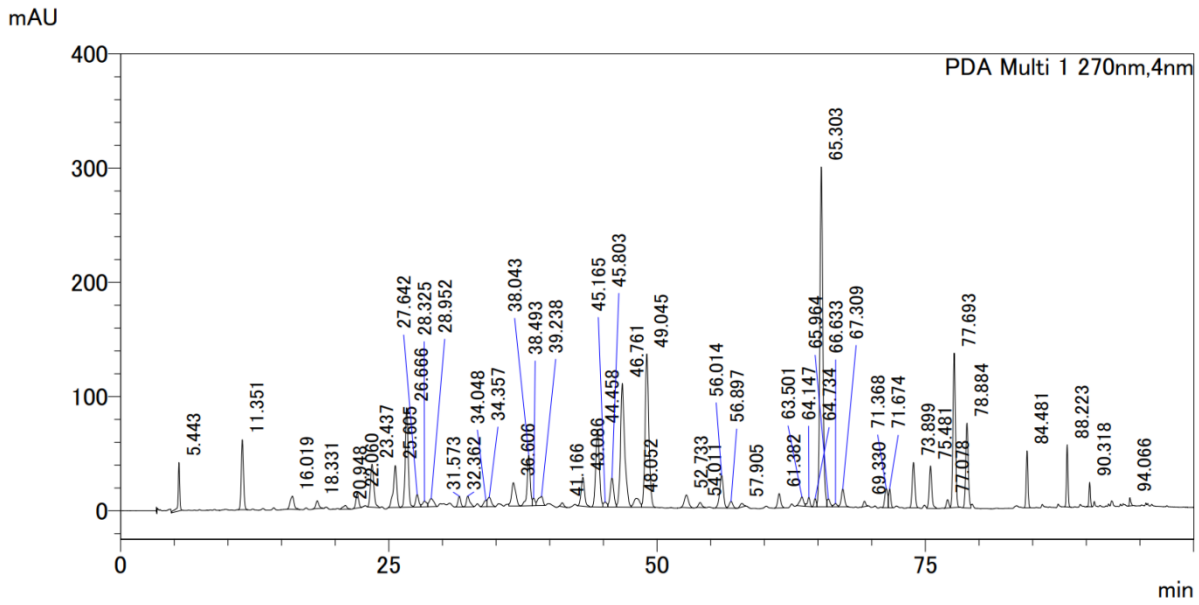

Figure S2.19 HPLC chromatogram of the ethyl acetate fraction of DH and HQ methanol extract (DHHQM-EA)

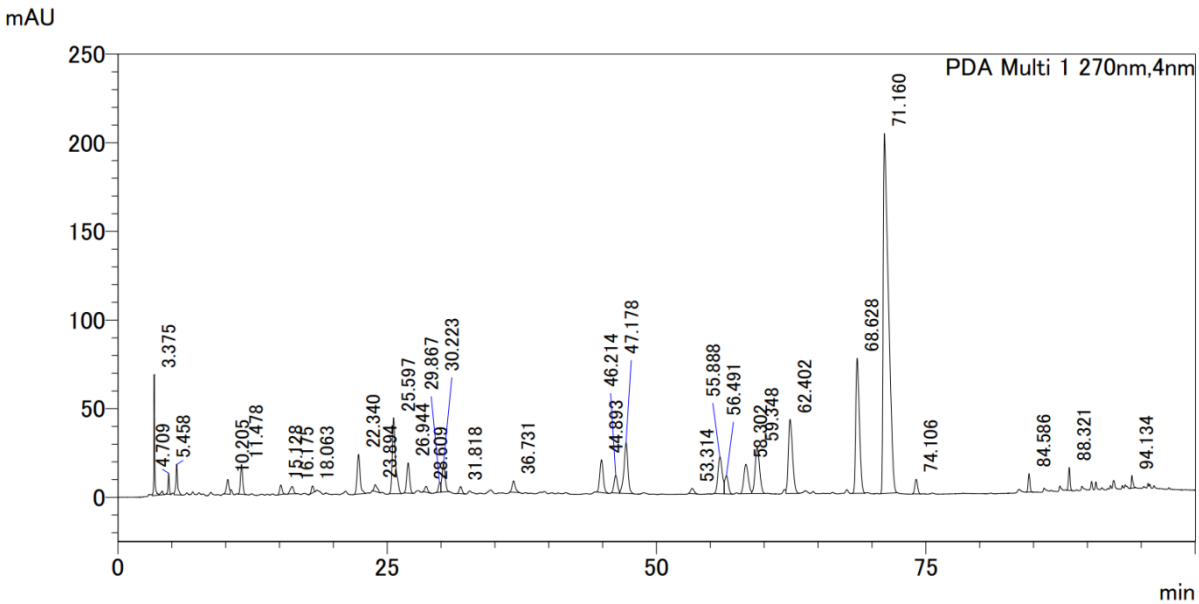

Figure S2.20 HPLC chromatogram of the methanol extract of DH and CHL (DHHLM)

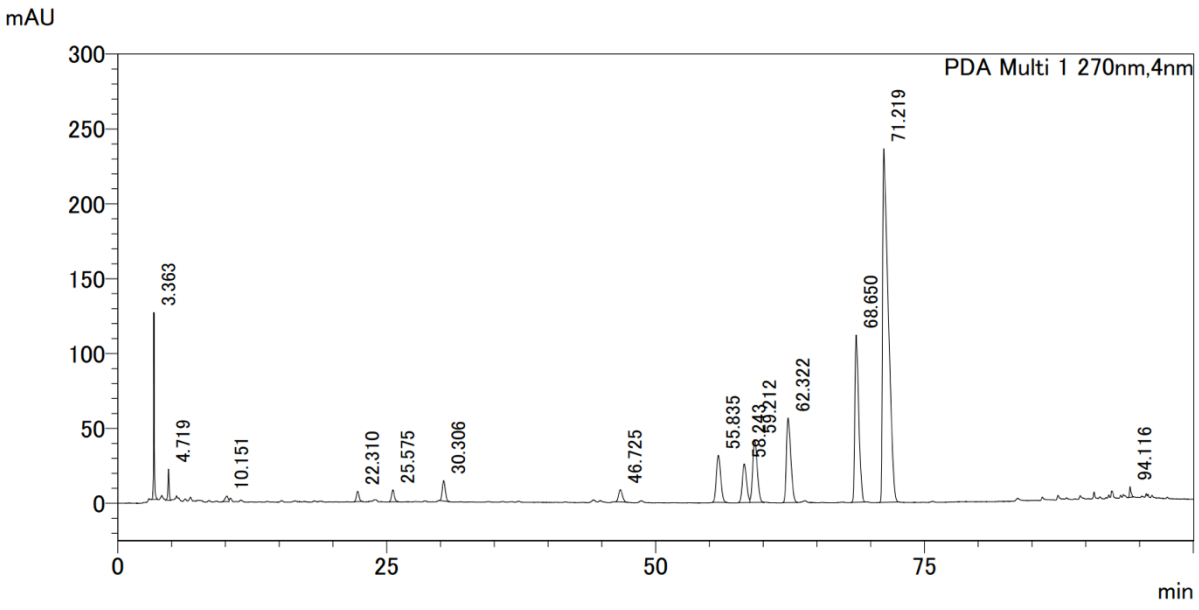

Figure S2.21 HPLC chromatogram of the water fraction of DH and CHL methanol extract (DHHLM-W)

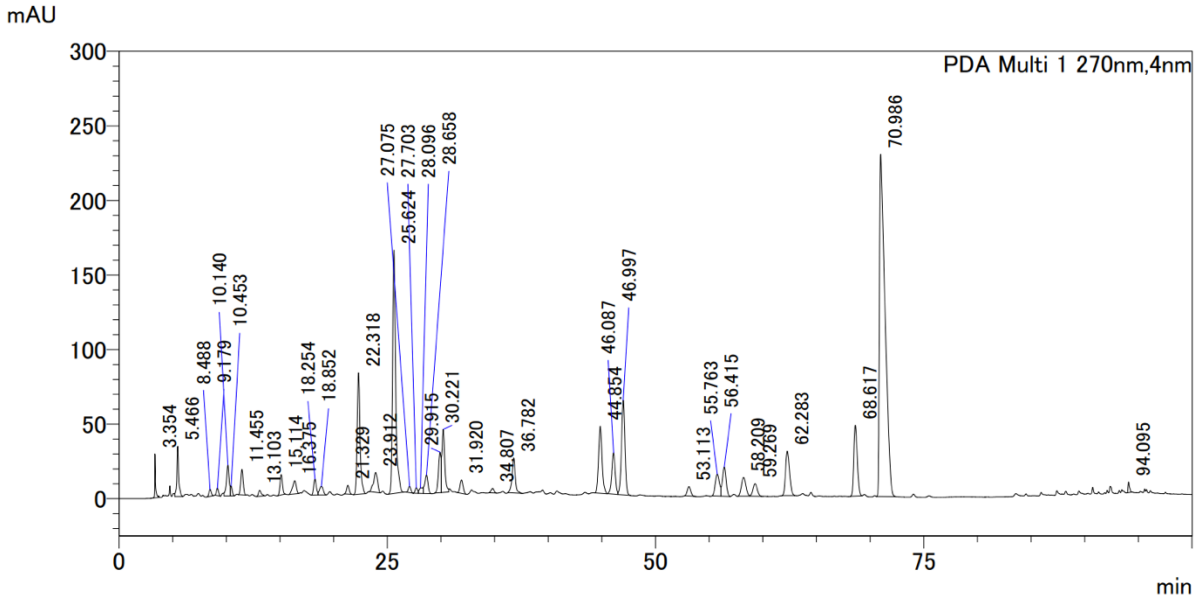

Figure S2.22 HPLC chromatogram of the *n*-butanol fraction of DH and CHL methanol extract (DHHLM-Bu)

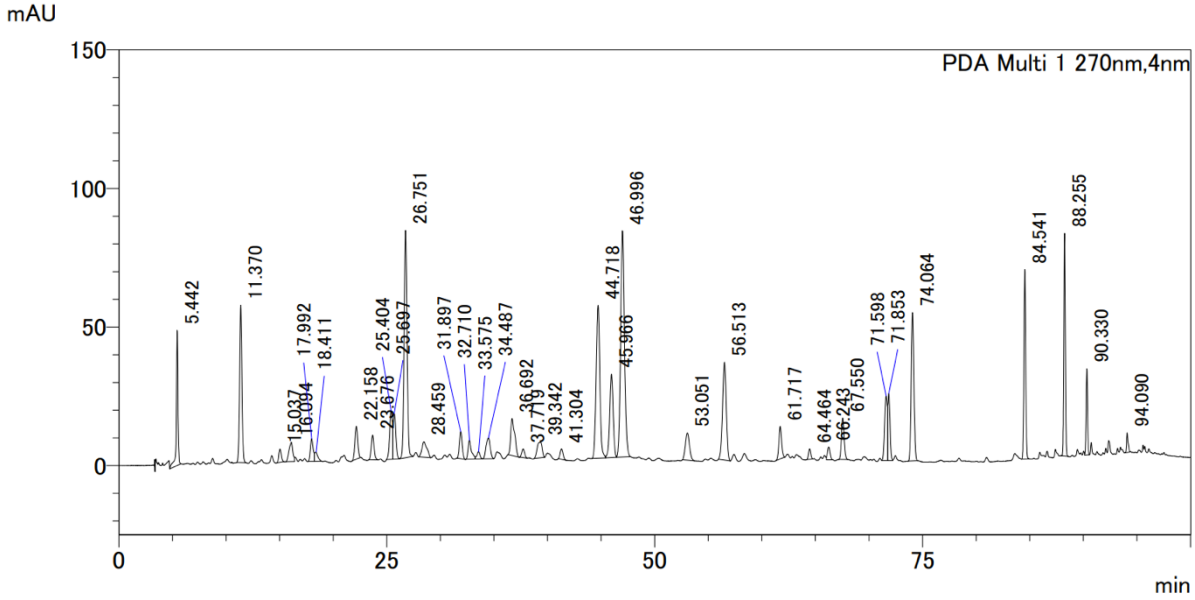

Figure S2.23 HPLC chromatogram of the ethyl acetate fraction of DH and CHL methanol extract (DHHLM-EA)

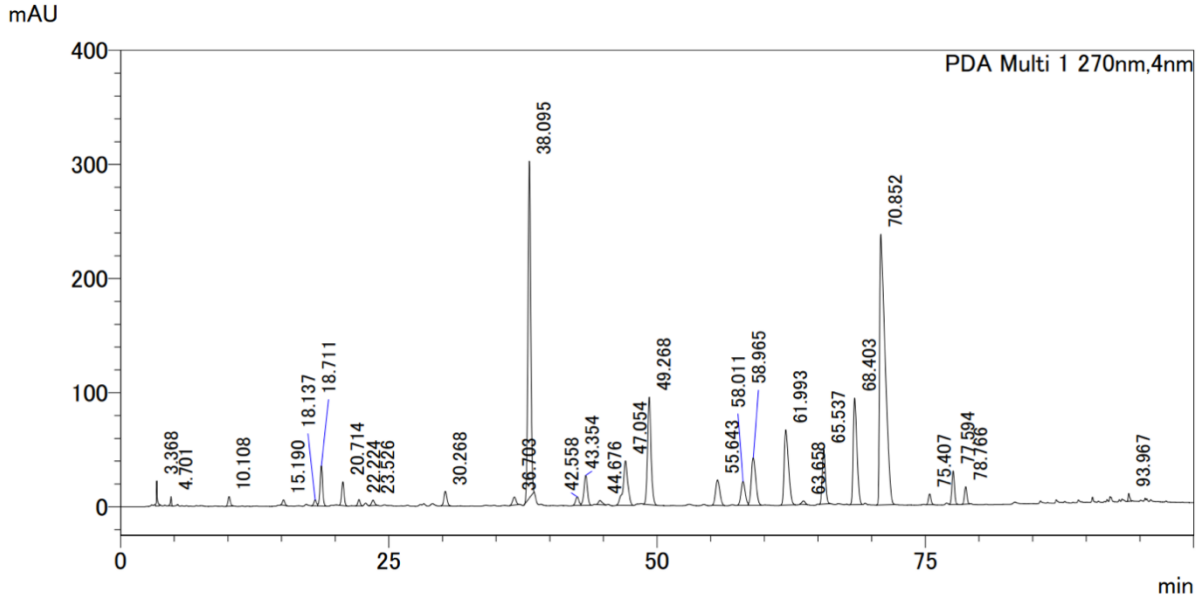

Figure S2.24 HPLC chromatogram of the methanol extract of HQ and CHL (HQHLM)

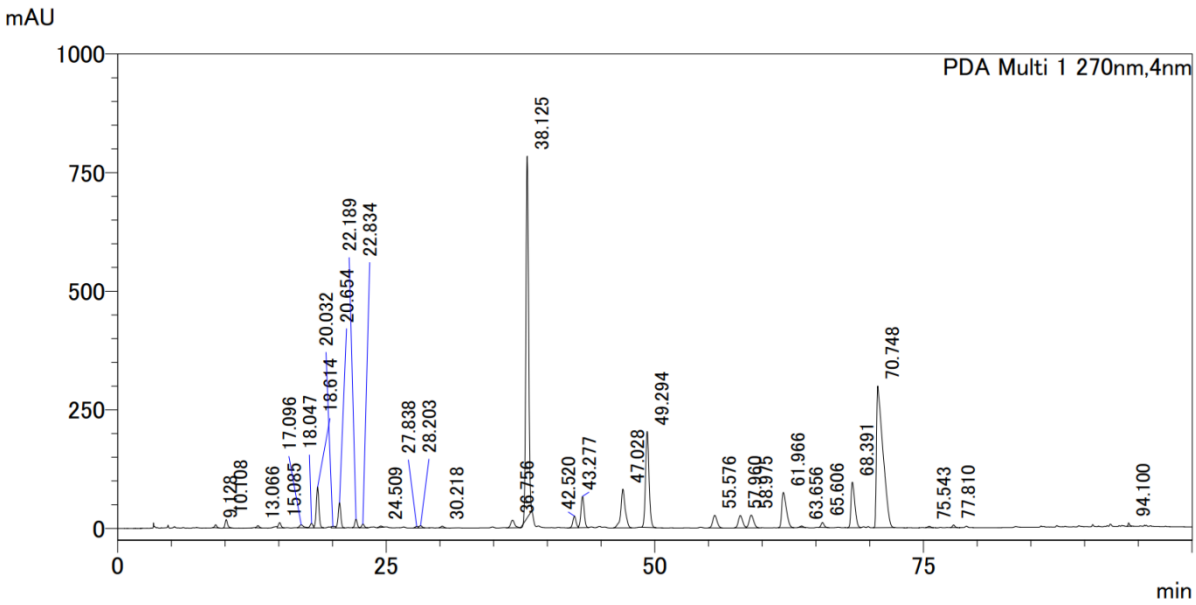

Figure S2.25 HPLC chromatogram of the water fraction of HQ and CHL methanol extract (HQHLM-W)

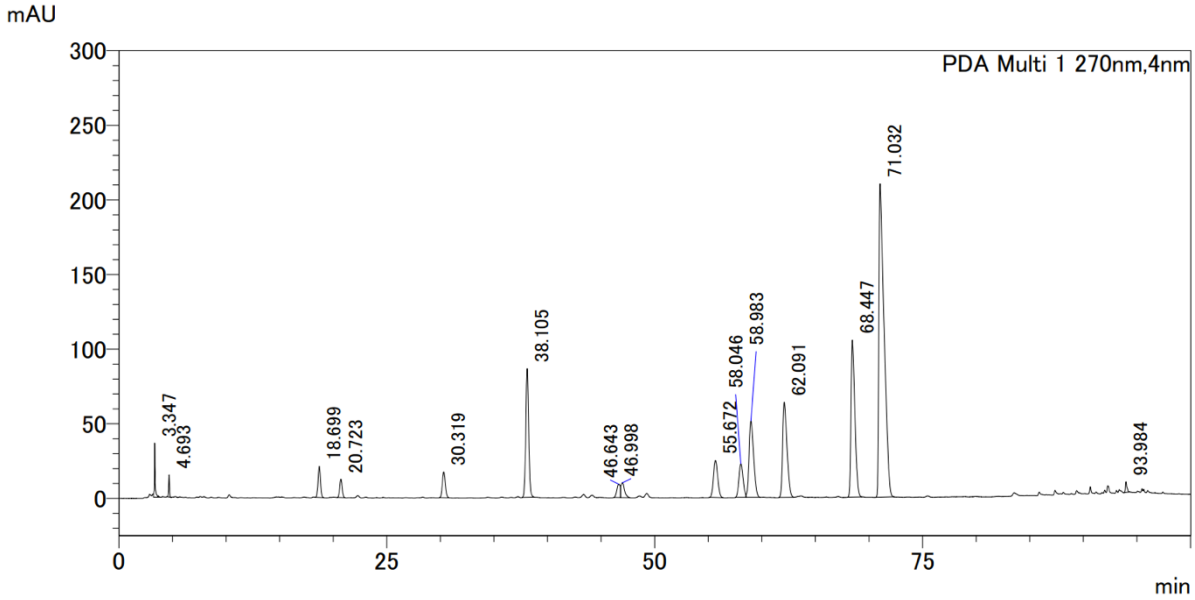

Figure S2.26 HPLC chromatogram of the *n*-butanol fraction of HQ and CHL methanol extract (HQHLM-Bu)

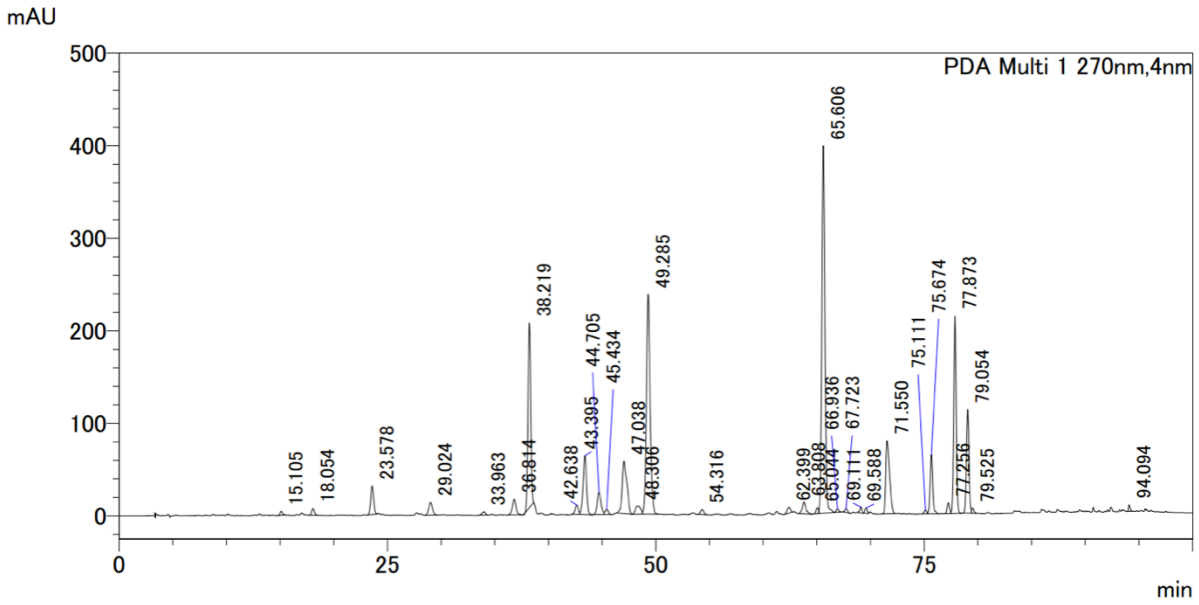

Figure S2.27 HPLC chromatogram of the ethyl acetate fraction of HQ and CHL methanol extract (HQHLM-EA)

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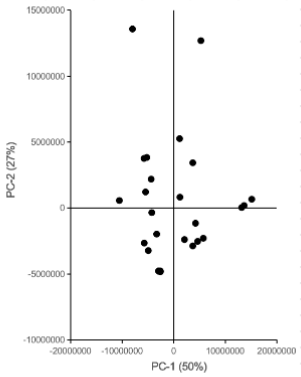
2		Peak No.	EC50	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
3	SHXXT-M	Area	16.15	230594	62813	115101	100303	185139	53368	69207	54438	436058	209226	338248	199840	651677	233221	64746	296855	241075	3863931	90547	368406	357505	143628	1264238	1384335	51189	423109	151558	360092	654664	1030615	680205	1355080	5415022	98901	95601	323929	172460	85715	108594
4	SHXXTM-W	Area	20.01	458349	126041	0	0	0	0	0	0	304584	174177	84393	0	151533	0	0	218644	0	991774	0	0	0	0	0	79611	0	493847	0	393859	841698	1097622	0	1586229	4938815	0	0	0	0	0	0
5	SHXXTM-Bu	Area	6.65	83957	140113	0	206954	98755	87477	93904	68337	734974	400007	802717	0	1302829	0	195089	429150	359892	8281831	206722	738291	279693	124329	1886120	1977930	0	363709	93625	348989	349837	842061	54793	1205141	6385395	0	0	0	0	0	0
6	SHXXTM-EA	Area	32.15	0	400759	0	0	599068	78876	206869	92765	0	0	331526	667462	294455	1062047	0	0	493241	614428	52441	348462	1463117	535604	2167621	2448285	174976	0	636684	0	0	177594	4424626	91612	0	503506	606641	1892565	1029899	686894	526174
7	CHLM	Area	10.79	188336	100414	0	244226	0	180245	0	207009	0	0	0	0	0	0	0	481027	0	0	0	0	0	0	322406	0	82637	1319034	0	1149241	2397953	3593413	0	4529627	16706289	0	0	0	0	0	0
8	CHLM-W	Area	8.28	268973	103881	0	71835	0	0	0	0	0	0	0	0	0	0	0	561246	0	0	0	0	69167	0	339603	0	0	1442854	0	1211259	2636113	3714194	0	4912842	16037568	0	0	0	0	0	0
9	CHLM-Bu	Area	11.05	74772	55075	100197	1057469	0	890278	0	1001175	0	0	0	0	0	0	0	0	0	0	0	0	0	0	79666	0	0	720023	0	740412	576959	2116450	0	2363654	19354611	0	74412	0	0	0	0
10	CHLM-EA	Area	104.18	52613	0	0	0	0	89376	0	232521	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	81153	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	HQM	Area	15.8	0	0	0	0	0	0	0	0	1082084	646660	164938	264716	0	0	0	0	333670	7919887	240557	907790	229400	0	1710620	3453591	0	0	0	0	0	0	2274898	0	0	0	286864	960893	495479	0	0
12	HQM-W	Area	15.33	0	0	0	0	0	0	0	0	709500	440469	65630	0	0	0	0	0	171105	7136885	209635	437046	0	0	905579	1515673	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	HQM-Bu	Area	5.18	0	0	0	56886	0	73517	0	0	3571275	2125948	778637	52698	66303	58991	0	0	602353	16760086	419803	2281016	50827	0	4265655	7285245	0	0	0	0	0	0	367258	0	0	0	134231	293553	152205	0	0
14	HQM-EA	Area	23.28	0	0	0	0	0	0	0	0	0	0	0	1973433	0	0	0	93044	548271	2230821	222808	1535449	1737793	0	2811580	7957100	0	0	0	0	68611	0	17283773	0	0	0	2106468	7084424	3892428	0	0
15	DHM	Area	-	265623	0	196287	0	298432	0	124724	0	76740	0	76740	160758	1152668	393115	104478	275424	210121	0	0	0	494597	244318	730543	0	81926	0	253905	0	0	0	0	0	0	176592	0	0	0	144788	159031
16	DHM-W	Area	-	459801	95599	76373	0	0	0	0	0	0	0	133874	120104	574633	0	58110	135332	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	DHM-Bu	Area	-	0	0	237012	0	188096	0	147782	0	0	0	1016315	164720	1655119	0	211521	509386	257344	0	0	0	612916	335211	741438	0	70991	0	244478	0	0	0	0	0	0	0	0	0	0	0	0
18	DHM-EA	Area	12.76	0	0	349931	0	928600	0	286804	0	127687	0	219619	160137	775817	1771926	0	0	346364	0	0	0	1193300	569050	2100833	0	296891	0	740681	0	0	0	0	0	0	854864	0	0	0	730557	709520
19	HQHLM	Area	6.88	108689	52182	0	125871	0	74637	0	88067	578648	337184	84422	92917	0	0	0	244335	155471	5397723	161797	589047	92210	0	1178910	2150678	0	616583	0	567965	1213883	1862589	963638	2244259	8447799	0	151326	477560	240837	0	0
20	HQHLM-W	Area	5.03	0	0	0	278376	0	184596	0	162973	1451430	884313	279584	0	0	0	0	60264	406472	13433326	503187	1374472	0	0	2320396	4597380	0	735739	0	725173	771407	2192347	202663	2372380	12429937	0	50954	84581	0	0	0
21	HQHLM-Bu	Area	15.89	162746	77369	0	0	0	0	0	0	333430	197369	0	0	0	0	0	332410	0	1610500	0	0	0	0	234592	0	0	687983	0	606641	1522742	1807463	0	2632075	7101713	0	0	0	0	0	0
22	HQHLM-EA	Area	15.93	0	0	0	0	0	68315	0	127357	0	0	0	598177	0	0	0	0	409114	3568327	205444	1395916	591535	0	1716351	5721906	0	0	0	0	0	135777	7771489	0	1948972	0	1067688	3465597	1830700	0	0
23	DHHQM	Area	33.79	237077	51031	155277	0	237129	0	87673	0	588447	285574	448835	214325	804951	303350	79659	218359	319110	4151525	103266	465174	471055	181254	1460660	1757582	63629	0	183744	0	0	0	951206	0	0	125871	128068	440079	235117	113550	126159
24	DHHQM-W	Area	24.35	280401	57812	0	0	0	0	0	0	368372	215629	116039	87058	499986	0	166966	0	86450	2927707	72481	145316	0	0	364274	544765	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25	DHHQM-Bu	Area	17.01	89226	0	215038	0	200289	0	161038	0	1224855	640531	1460646	272700	1217211	0	254286	397142	590156	8068352	192236	1054036	677120	351778	2712521	3161720	71046	0	275772	0	0	0	121058	0	0	0	118106	198817	105193	97922	100765
26	DHHQM-EA	Area	28.75	0	0	441910	0	911382	0	290122	0	133897	0	231842	834153	808759	1660419	0	0	552479	890504	0	569537	1636903	588150	2925248	3241396	297924	0	697393	0	0	0	5800879	0	0	676932	626604	2205475	1226916	568223	537502
27	DHHLM	Area	13.94	325681	88821	191771	137671	243726	83027	87051	58566	0	0	430732	0	906124	316968	77732	322034	130714	0	0	0	415643	217226	726720	0	64194	579656	235334	470826	757700	0	0	1799900	6811370	144549	0	0	0	118377	134971
28	DHHLM-W	Area	15.48	512928	154386	0	69609	0	0	0	0	0	0	120790	0	144318	0	0	275075	0	0	0	0	0	0	203510	0	0	887032		706097	1231620	0	0	2843007	8731318	0	0	0	0	0	0
29	DHHLM-Bu	Area	14.12	140300	0	385254	362981	266072	216515	213596	186899	0	0	1529142	0	3255469	69956	443695	793967	483808	0	0	0	1032738	613017	1423195	0	137822	395382	466293	363649	234573	0	0	1062445	8269240	0	0	0	0	0	0
30	DHHLM-EA	Area	17.42	0	0	498274	0	853501	76866	171316	66796	0	0	214729	0	310603	1551164	0	0	347814	0	0	0	1322774	673866	2010605	0	262753	0	849432	0	0	0	0	0	0	933495	0	0	0	781181	792611

Figure S3.1 The pretreatment of HPLC data of all samples and fractions

1	Area																																								
2	ローディング	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
3	PC-1	0.008834695	0.002802	-0.00349	0.021707	-0.01249	0.014805	-0.0039	0.0161988	-0.031584	-0.01756	-0.01066	-0.03307	-0.00726	-0.02232	-0.00049	0.015753	-0.02007	-0.18992	-0.00653	-0.04496	-0.04148	-0.00861	-0.09624	-0.19262	-0.00279	0.059762	-0.0099	0.053144	0.091723	0.139688	-0.25815	0.200396	0.866586	-0.01046	-0.03189	-0.10834	-0.05923	-0.01042	-0.00956
4	PC-2	-0.01101083	-0.00306	-0.0136	0.008524	-0.02503	0.004888	-0.00755	0.002076	0.1315848	0.079228	0.015846	-0.004	-0.04013	-0.04243	-0.00238	-0.00524	0.016693	0.870239	0.026187	0.102762	-0.03691	-0.01995	0.124667	0.324343	-0.00821	0.012848	-0.02393	0.013972	0.013042	0.060133	0.016477	0.043314	0.268237	-0.0217	0.005723	0.01229	0.005069	-0.01904	-0.01813
5	PC-3	-0.007414639	0.001395	-0.00599	0.007475	-0.00524	0.006545	-0.00277	0.0084196	-0.057712	-0.03332	-0.03199	0.081232	-0.04301	-0.00329	-0.00673	-0.00368	0.010374	-0.22043	0.001917	0.042105	0.061709	-0.00694	0.046931	0.278692	-0.00102	0.016048	-0.00616	0.014503	0.028649	0.0502	0.785866	0.055391	0.287163	-0.00324	0.096183	0.32267	0.176523	-0.00207	-0.00336
6	PC-4	-0.05348933	-0.006	0.10356	0.065226	0.176793	0.048769	0.067734	0.0398588	0.0216348	0.002115	0.230882	0.018132	0.410733	0.273116	0.042998	0.034789	0.115708	-0.06627	-0.00515	0.023737	0.308234	0.168855	0.509398	0.066641	0.058805	-0.05265	0.175784	-0.0366	0.18218	-0.18894	-0.0704	-0.21703	0.177083	0.129387	-0.00732	-0.03732	-0.01818	0.118602	0.112586
7	PC-5	0.02369497	-0.01877	-0.02136	0.100289	-0.15805	0.073339	-0.02462	0.0837258	-0.071611	-0.03555	0.158298	-0.01252	0.34746	-0.35856	0.067409	0.044837	-0.01343	0.046016	0.008974	0.002922	-0.13945	-0.06129	-0.31786	-0.04031	-0.04995	-0.09551	-0.11416	-0.07086	-0.30172	-0.39446	0.059851	-0.38253	0.153073	-0.1812	0.015918	0.035108	0.017099	-0.16425	-0.15381
8	PC-6	0.05788706	0.003864	0.001318	-0.12342	-0.06938	-0.13726	0.014113	-0.167478	0.0257113	0.009621	0.322873	0.030324	0.536349	-0.23427	0.086835	0.257513	0.041997	0.011419	-0.00993	0.004926	0.04332	0.024479	0.064883	-0.04325	-0.01303	0.131038	-0.02812	0.09002	0.322799	0.108651	0.044591	0.438636	-0.14482	-0.1274	-0.00613	0.019021	0.015151	-0.10845	-0.10185
9	PC-7	-0.09366531	-0.00421	0.0117	0.002873	0.071636	-0.01899	0.027668	-0.019788	-0.375675	-0.23048	-0.04766	0.059596	0.152943	0.099512	0.028831	0.017028	0.015927	0.334349	0.029673	-0.19392	0.145264	0.051621	-0.13496	-0.55526	0.016715	-0.05187	0.064942	-0.01787	-0.08818	0.384591	0.236584	-0.13759	-0.01437	0.059679	-0.00227	0.039221	0.02286	0.051958	0.049783
10									-20000000	0							-0.4	0																							
11									20000000	0							1	0																							
12	スコア								0	15000000							0	-0.2																							
13		PC-1	PC-2	PC-3	PC-4	PC-5	PC-6	PC-7	0	-10000000							0	1																							
14	SHXXT-M	1223758	822100.9	-670358	-18792	-91400.6	212012.2	280945.4																																	
15	SHXXTM-W	2098075	-2393009	-1267563	-1330403	-56332	96162.16	-83026.7																																	
16	SHXXTM-Bu	1154769	5263878	-1900463	665804	683407.1	524512.5	945986.3																																	
17	SHXXTM-EA	-4922871	-3229232	2480250	856896.6	-984625	-379057	58778.51																																	
18	CHLM	1.37E+07	189014.1	2718554	-513711	-1093722	488625.2	-59472.5																																	
19	CHLM-W	1.32E+07	35234.3	2563581	-794921	-1531782	976584.2	-77428.2																																	
20	CHLM-Bu	1.51E+07	665039.3	3232435	1072959	1615515	-2184970	-161200																																	
21	CHLM-EA	-2580231	-4780105	-2608695	-1454154	481854.2	-636193	-280157																																	
22	HQM	-5748670	3764147	-1135080	-903095	212654.5	-286012	189055.5																																	
23	HQM-W	-4359996	2191079	-3778895	-1320169	381879.9	-459208	756087.9																																	
24	HQM-Bu	-7986115	1.36E+07	-3867736	488156	-584449	-4238.93	-1396220																																	
25	HQM-EA	-1.05E+07	570724.6	1.63E+07	-561792	444613.1	440014.2	262797.7																																	
26	DHM	-2698881	-4819502	-2622699	-88346.5	261076	71078.63	7821.152																																	
27	DHM-W	-2573558	-4835430	-2657330	-1211323	694674.9	-162444	-174106																																	
28	DHM-Bu	-2701732	-4808980	-2658518	241832.2	813916.1	861243.7	49416.68																																	
29	DHM-EA	-2935967	-4776810	-2521473	1607359	-1402566	-632732	192168																																	
30	HQHLM	3713994	3432572	489654.1	-680551	-455020	90878.91	226048.6																																	
31	HQHLM-W	5287908	1.27E+07	-212353	387885.5	-85884.5	-497153	670331.8																																	
32	HQHLM-Bu	4241503	-1155460	-666260	-1438269	-757281	513288	156158.4																																	
33	HQHLM-EA	-5444964	1217383	6623130	-567918	651602.6	-478986	-537763																																	
34	DHHQM	-4250066	-347728	-2079721	-73165.4	192158	119444.6	129003.3																																	
35	DHHQM-W	-3295529	-1975266	-3152462	-1195327	628436.5	-196725	246658.8																																	
36	DHHQM-Bu	-5212196	3836540	-3301996	963674.8	208592.8	881717.8	-100992																																	
37	DHHQM-EA	-5701041	-2658070	3879077	1779969	-1227562	-234200	25581.65																																	
38	DHHLM	3715151	-2875124	-541434	441763.2	369228.7	210973.3	-516507																																	
39	DHHLM-W	5757196	-2293956	85397.23	-650427	229401.2	196461.1	-962419																																	
40	DHHLM-Bu	4654084	-2531684	-248097	2871186	1911738	1355530	-43999.2																																	
41	DHHLM-EA	-2915934	-4788354	-2503560	1404321	-1493784	-882430	182815																																	



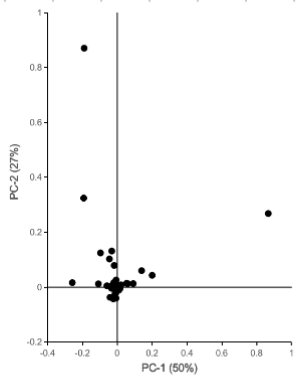
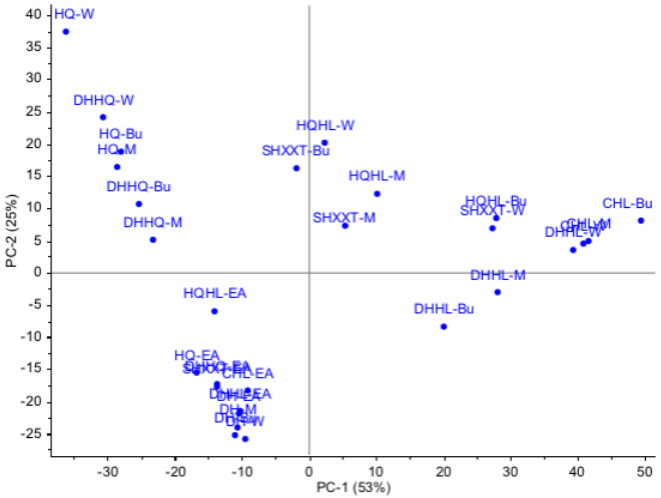
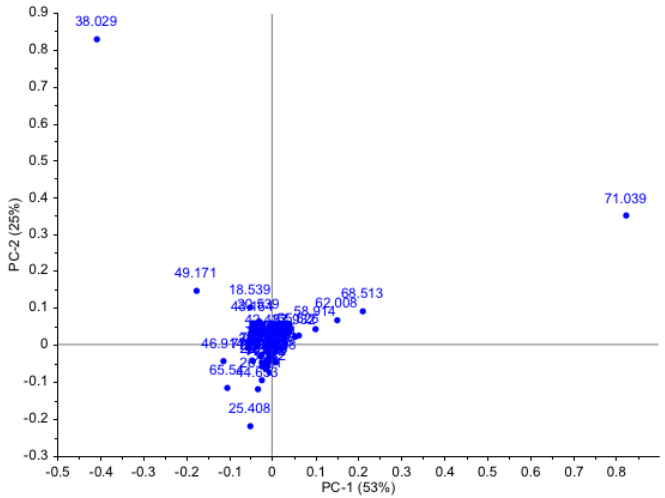


Figure S3.2 The results of PCA calculation (by Area)

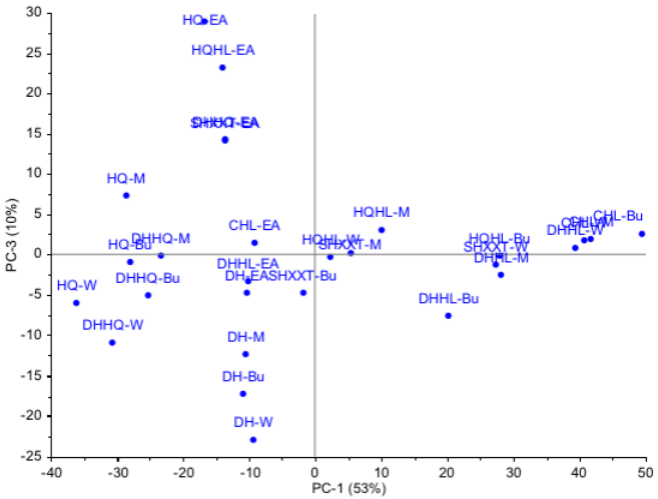
Score



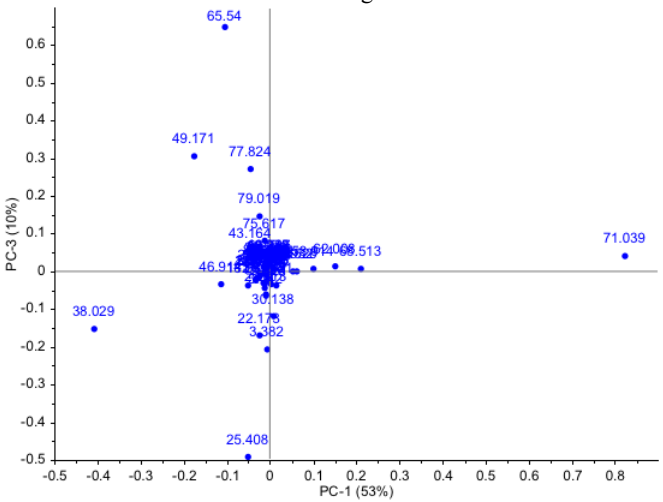
Loadings



Score



Loadings



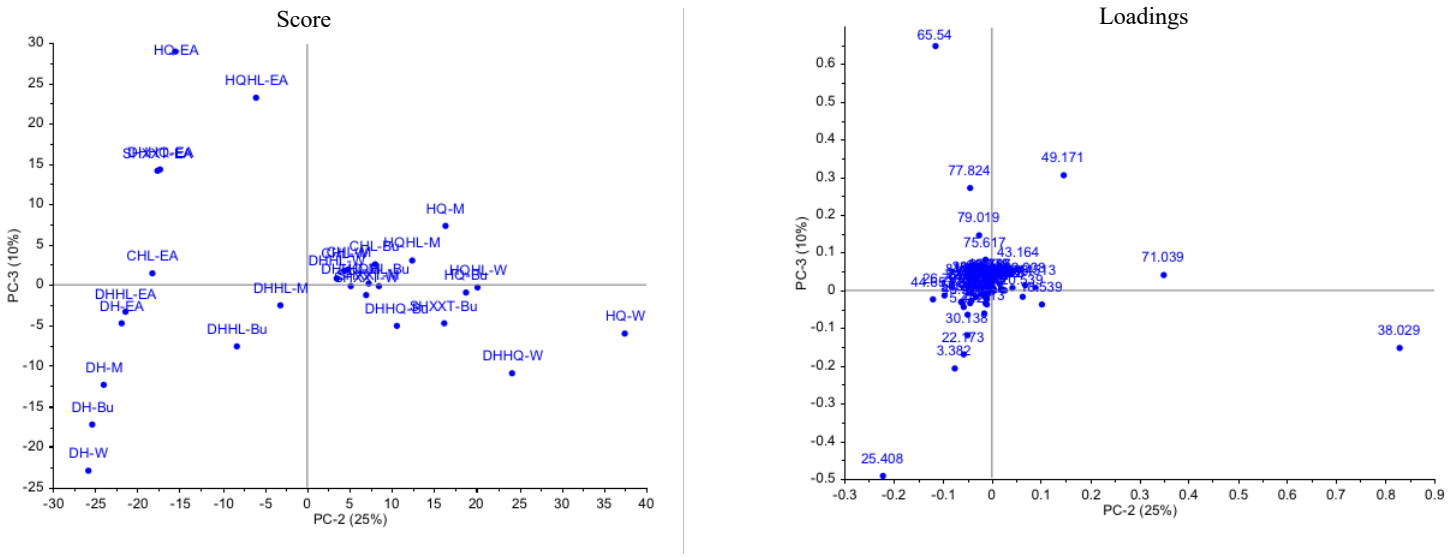
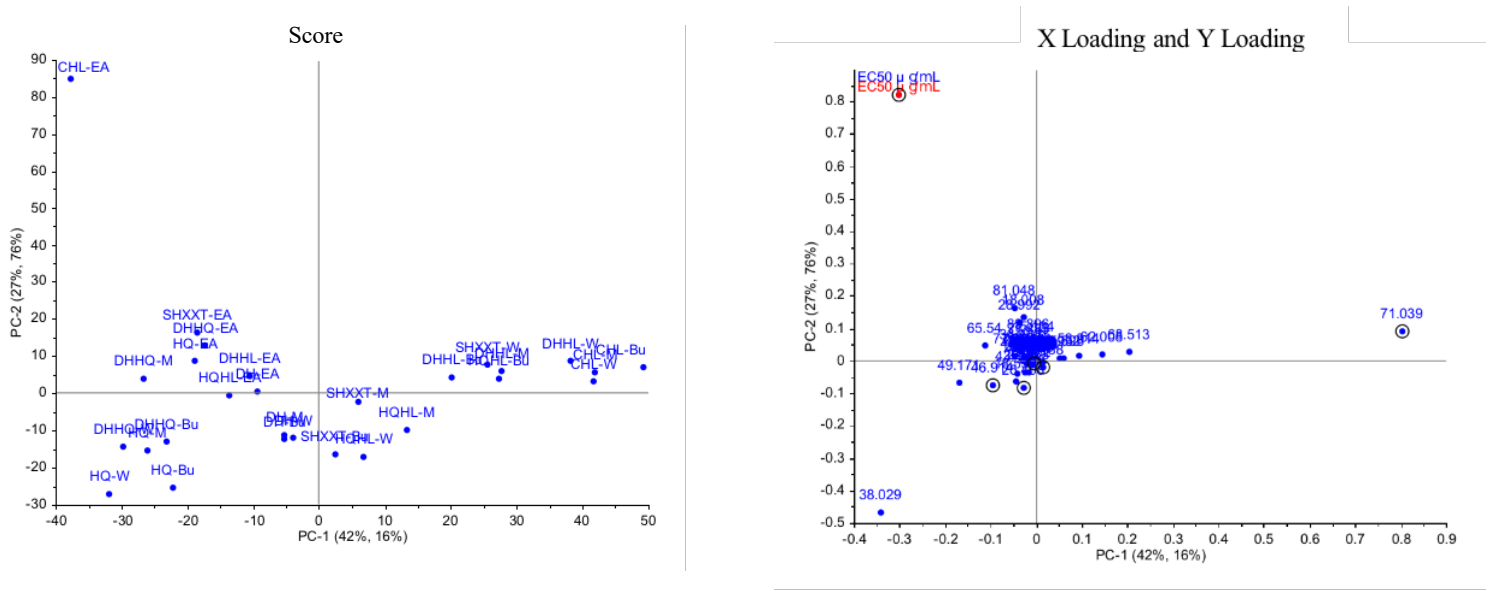
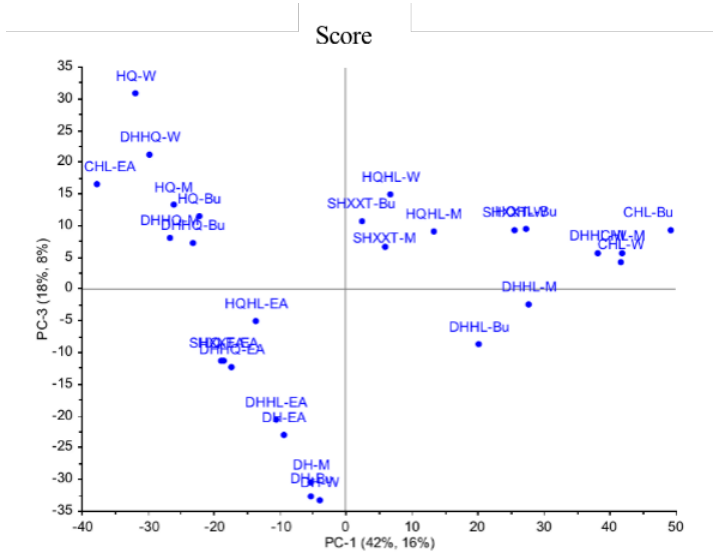
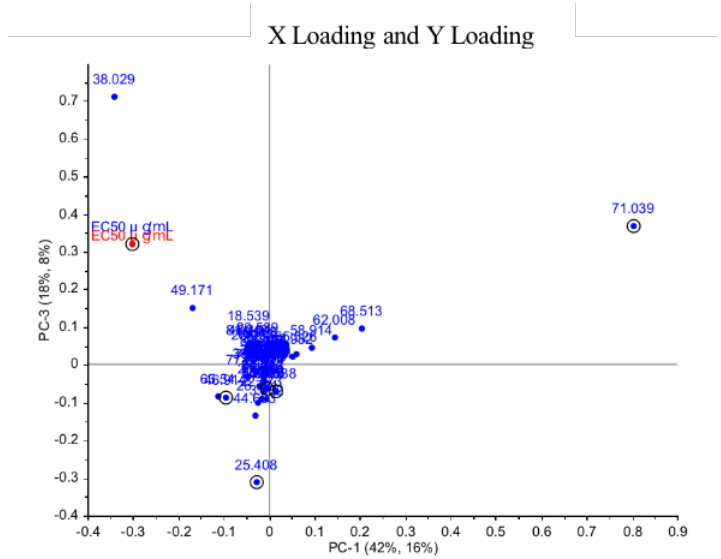
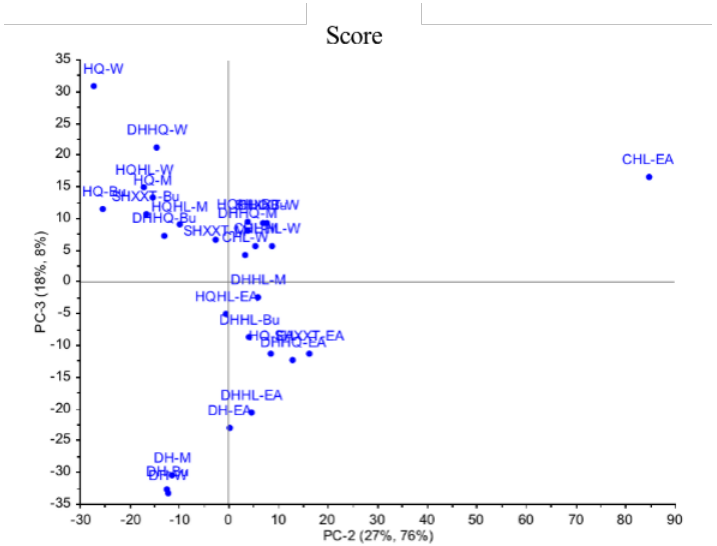


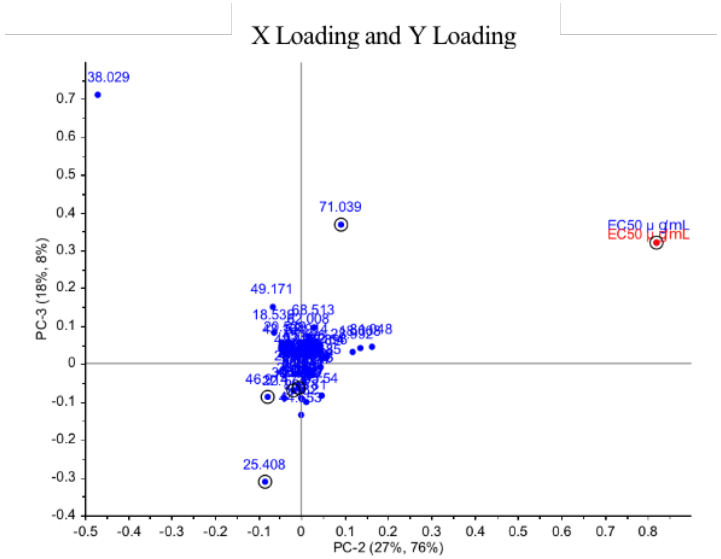
Figure S4 The results of PCA calculation (by Area%)

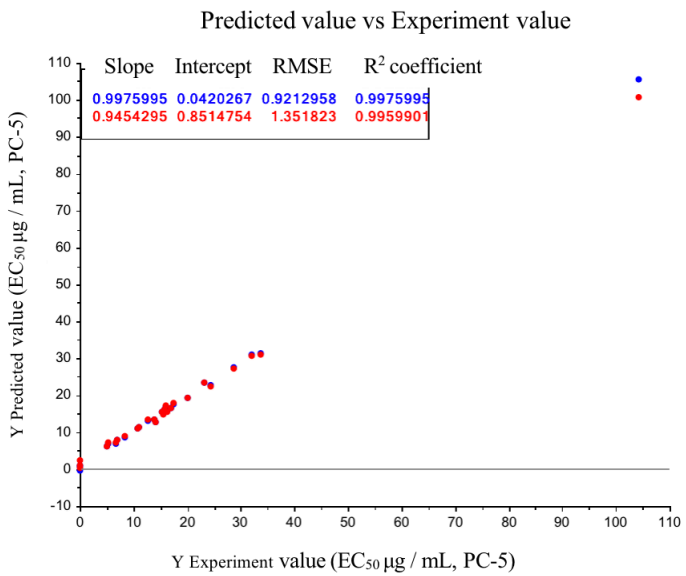



Score
HQ-W
DHHQ-W
CHL-EA
HQ-M
HQ-Bu
DHHQ-M
DHHQ-Bu
HQHL-W
SHXXT-Bu
HQHL-M
SHXXT-M
CHL-Bu
DHHCHL-M
CHL-W
DHHL-M
HQHL-EA
DHHL-Bu
DHHQ-EA
DHHL-EA
DH-EA
DH-M
PC-3 (18%, 8%)
PC-1 (42%, 16%)


X Loading and Y Loading
38.029
71.039
EC50 μ g/mL
EC50 μ g/mL
49.171
18.539
68.513
62.008
25.408
PC-3 (18%, 8%)
PC-1 (42%, 16%)


Score
HQ-W
DHHQ-W
CHL-EA
HQHL-W
HQ-M
HQ-Bu
SHXXT-Bu
HQHL-M
DHHQ-M
DHHQ-Bu
CHL-W
DHHL-M
HQHL-EA
DHHL-Bu
DHHQ-EA
DHHL-EA
DH-EA
DH-M
PC-3 (18%, 8%)
PC-2 (27%, 76%)


X Loading and Y Loading
38.029
71.039
EC50 μ g/mL
EC50 μ g/mL
49.171
68.513
25.408
PC-3 (18%, 8%)
PC-2 (27%, 76%)



RMSE: root-mean-square error

Figure S5 The results of PCR calculation (by Area%)

Publications

This thesis contains the whole contents of the following papers:

1) Jianbo Wu, Souichi Nakashima, Marina Shigyo, Mutsumi Yamasaki, Sumire Ikuno, Aoi Morikawa, Shigehiko Takegami, Seikou Nakamura, Atsuko Konishi, Tatsuya Kitade, Hisashi Matsuda. Antihypertensive constituents in Sanoshashinto. *J. Nat. Med.* 74: 421–433 (https://doi.org/10.1007/s11418-019-01382-9) [Chapter I, II, III]

2) Jianbo Wu, Souichi Nakashima, Seikou Nakamura, Hisashi Matsuda. Effects of Sanoshashinto on left ventricular hypertrophy and gut microbiota in spontaneously hypertensive rats. *J. Nat. Med.* 74: 482–486 (https://doi.org/10.1007/s11418-020-01387-9) [Chapter III]