# DOCTORAL THESIS

**Pharmacological characteristics of medicinal plant extracts on porcine basilar artery**

**(**薬用植物抽出液のブタ脳底動脈における薬理学的研究**)**

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# Contents

## ABSTRACT

The present thesis was designed to study the effects of extracts from medicinal plants, including *Artemisia vulgaris* L. leaf (AVL), *Gingko biloba* leaf (GB) and *Sophora japonica* L. flower bud (SJ), on the responsiveness of porcine basilar artery (PBA), in order to explain the therapeutic applications of them in Vietnamese traditional medicine.

The first study characterized the responsiveness of AVL on isolated PBA. In Vietnamese herbalism, there are conflicting opinions about the effect of AVL (English name: mugwort) on hypertension. Some ethnic doctors recommend the use of AVL for treatment of hypertension, whereas others advise against it. The purpose of the first study was to clarify the pharmacological characteristics of AVL in isolated arteries to explain the conflicts surrounding the use of AVL for treatment of hypertension. The effects of the AVL extract on PBA was examined in the two different conditions, including the resting tension and the pre-contracted tension. We observed that depending on arterial conditions, AVL had the two opposite effects, as it induced contraction on artery of resting tension, but induced relaxation on artery of KCl pre-contraction. AVL -induced contraction was inhibited by methiothepin (a 5-$HT_1$ and 5-$HT_2$ receptor antagonist) in the absence of ketanserin (a 5-$HT_2$ receptor antagonist) and was competitively inhibited by the mixture of these two antagonists, which was similar to the contraction induced by agonist 5-HT on PBA. HPLC analysis revealed that the retention time of the first peak in the AVL profile was similar to that of the 5 -HT standard, and that addition of 5-HT to the AVL sample enhanced this peak. On the other hand, AVL induced endothelium -independent relaxation under precontracted conditions

with 60 mM KCl. In $Ca^{2+}$-free 60 mM KCl-containing solution, pretreatment with AVL significantly inhibited $CaCl_2$-induced contraction. This study, for the first time, demonstrated that AVL has two opposite effects, contraction and relaxation, on isolated artery, which may help to explain the conflicting indications for AVL in traditional herbalism. In addition, the results identified that 5-HT is a significant factor affecting artery contraction in the presence of AVL.

The second study characterized the responsiveness of SJ and GB on isolated PBA. In Vietnamese herbalism, GB and SJ are described as plants used for the treatment of many cerebral disorders, including those are associated with cerebral vasospasm. The aim of the second study was to evaluate, for the first time, the antagonistic effects of GB and SJ extracts on cerebral vasoconstriction in response to KCl, extracellular $Ca^{2+}$, histamine, 5-hydroxytryptamine (5-HT), 9,11-dideoxy-9á,11á-methanoepoxy prostaglandin (PG) $F_{2á}$ (U46619) and bradykinin (BK), with the view of explaining their traditional application for diseases that associated by cerebral vasospasm. Isolated PBA and endothelial cells from them were used as the study materials. Neither SJ nor GB had any effect on the contractions induced by KCl and extracellular $Ca^{2+}$. SJ significantly inhibited the contraction induced by histamine, 5-HT, U46619 and BK, whereas GB inhibited histamine-induced contraction, but had no effects on the contractions induced by 5-HT, U46619 and BK. In the presence of diphenhydramine (a $H_1$ receptor antagonist), ketanserin (a 5-$HT_2$ receptor antagonist) and ONO-3708 (a thromboxane (TX) $A_2$/PG receptor antagonist), the inhibitory effects of these extracts on the contractions induced by histamine, 5-HT and U46619 were abolished. SJ significantly inhibited the contractions induced by BK and $PGF_{2á}$, but in

the presence of ONO-3708 ($10^{-5}$ M) had no effect on them. BK enhanced the production of $PGF_{2\acute{a}}$ from cultured PBA endothelium cells, and SJ significantly attenuated this enhancement. These results suggest that SJ and GB have $H_1$-antagonistic effect, and that SJ also attenuate cerebral vasoconstriction mediated via 5-$HT_2$ and $TXA_2$/PG receptors, and therefor partly explain their traditional therapeutic used for diseases related to cerebral spasm. In addition, these findings appear to explain why SJ has been recorded as a reputed therapeutic medication applied for the cerebral hemorrhages.

In conclusions, the present thesis verified the effects of AVL, SJ and GB on PBA, and from that give pharmacological basis to explain the contraindications of AVL in hypertension, and the therapeutic uses of SJ and GB in cerebral hemorrhage and other diseases associated with cerebral vasospasm in traditional medicine. The results demonstrated pharmacological characteristics of those plants and partly explained their treatment effects, but further researches are necessary to support their potentials and propose the safe applications. In addition, our data have not addressed the *in vivo* results, so it remained to be determined with future researches. We expect that our next studies, which investigate the effects of those plants *in vivo* and in different pathological states would provide more evidences to exploit their therapeutic uses.

# General Introduction

Traditional medicine is used worldwide and it is of great economic importance in the 21st century [13]. Failure of many synthetic drugs and their side-effects have prompted many researches to go back to ancient healing methods which use herbal medicines, and many of thousands of plant species growing throughout the world have a direct pharmacological actions on the body [113]. Among the positive aspects of medicinal plants, researchers have mentioned diversity, flexibility, accessibility, prevalence in developing countries, increasing popularity in developed countries, relative low cost [94, 101], less dangerous side-effects, less residues, slower resistance [12].The traditional healers provide a popular and accessible service, especially in the rural areas where the sanitary situation is characterized by lack of qualified health workers, medicines and equipment [56]. As the access to conventional health care is often difficult, people use both conventional and traditional medicines to improve their health condition. According to a WHO report, about 70-80% of the world's population rely on nonconventional medicine mainly from herbal sources in their primary health care, especially in the developing countries where the cost of consulting a western style doctor and the price of medication are beyond the means of most people [18, 117]. Thus, traditional medicine is an important factor and remains the main resource for the majority of people for treating health problems, particularly in rural areas [111].

Most synthesis drugs are produced by imitating the herbal medicines, but they are produced artificially in pharmaceutical laboratories. At least one third of all used products

have plant origin. In former times, natural products were the origin of all medicinal drugs; however, in the last century, synthetic chemistry and biotechnology techniques have offered alternatives to natural sources [46]. Nonetheless, the decreasing efficacy of synthetic drugs and the increasing contraindications of their usage make the usage of natural drugs topical again, and the past few decades have witnessed a significant renewed interest for the field of natural medicine, including the medicinal plants. This, coupled with the application of new technologies to in research, has led to the discovery of relevant new natural-derived active compounds. Information presented sources of new drugs from 1981 to 2007 indicates that almost half of the drugs approved since 1994 have been based on natural products [47]. In many cases, modern Western science has corroborated the proper use of diverse traditional ethno-medicinal plants, such as ginkgo, ginseng, and centella, which have become a part of many modern therapies after thorough investigations establishing their quality, security, and safety [105].Medicine plants are now considered as a good source for the development of new drugs [2, 7, 8, 12, 31, 39, 40, 41], with promising effects in a wide variety of diseases such as cancer, diabetes, atherosclerosis and cardiovascular diseases, learning and cognitive complication, wounds, prevention and treatment of the toxicity induced by other drugs or toxin, and also possess many other health beneficial effects [7]. With regards to the beneficial phytochemicals in pharmaceuticals industry, the research on medicinal plants particularly are as important as the research on conventional drugs [6].

Healing with medicinal plants is as old as mankind itself [102]. Ethnomedicine is based on ancient written sources along with knowledge and practices that have been handed

down orally over the centuries, and traditional medicines that make use of herbal drugs are used throughout the world in accordance with practices that have been developed following specific rules over the centuries[105]. One way of introducing new ethno-medicinal plants into modern therapies is through studies of known species used in traditional herbalism around the world. Plants with a therapeutic use in folk medicine against specific diseases could be of great interest for researchers in the field of medicinal plants, as the studies about them would not only help to exploit their applications as useful therapies but might also lead to the discovery of new drugs. The review of local flora and its ethnobotanical use could thus lead to characterizations and localizations of numerous new, potential medicine plants. At first, it would be justified to research about plants that are recommended by local herbalists or written in the reference books of ethnic medicine, which were usually from ancient time and had been passed down to us through many generations [11], and modern researchers now consider that serious studies into the old manuscripts on medicinal plants would be a potential sources of contemporary pharmacotherapy [102]. In our study, we mainly used two books, in which the authors have collectedthe knowledges of folk therapies from many arears in Vietnam for reference, including the book,*Nhung cay thuocva vi thuocthuong dung tai Vietnam*[28], which has been well recognized as the most sufficient dictionary about Vietnamese ethnic medicine, and the book, *Selected Medicinal plants in Vietnam*, Volume I, II, [73, 74],which is the official documents of medicine plants published by National Institute of Materia of Vietnam.

Arterial hypertension is most important diseases in developing countries [4]. It has been named the “silent killer”, and it is the major contributor or risk factor to

cardiovascular morbidity and mortality [38]. In 2000, 26.4 % of the world's population suffered hypertension and it is predicted that this rate would increase by 60% in 2025 [61]. This common and asymptomatic disease is curable and can be easily diagnosed, but it may have fatal consequences if left untreated [78], such as it can cause heart failure, coronary artery disease, angina, myocardial infarction, development of thrombosis, and cerebral hemorrhage [82].Many synthetic antihypertensive agents are used for the treatment of hypertension, but these drugs have a number of side effects like muscle cramps, dizziness, extreme tiredness, dehydration, blurred vision, abnormal heart rate, skin rash, vomiting, kidney failure, fever, headache, diarrhea and many others, and therefore the use of appropriate herbal medicine, associated with lifestylechanges wasrecommended as a safer therapy [117]. Herbal medicines are getting more importance in the treatment of high blood pressure[70], and many plants described in traditional herbalism are currently used to control blood pressure due to their relatively high effectiveness, slight side effects and lower prices[4].Unlike allopathic remedies, traditional medicines using herbs are usually considered as a safe and effective adjunctive therapeutic approach for the treatment of hypertension[70], and are usually free from any undesired effects [117]. However, serious side effects have been reported on some medicinal plants which are being used on large scale, and therefore indicates that herbal medicines should also be subjected to extensive scientific and pharmacological screening before being recommended as drugs[44]. In addition, because information on the biochemistry of most medicinal plants is unknown [106], and the information about their uses are various, and sometimes even conflicted according to the locals and the herbalists, it is necessary to study to research and

standardize their therapeutic uses [11]. In our first study, we focused on the case of *Artemisia vulgaris* L. (AVL, English name: mugwort), because in Vietnamese traditional herbalism, there are conflicting opinions about the effectof this planton hypertension, as some ethnic doctors recommend the use of AVL for treatment of hypertension, whereas others adviseagainst it. Therefore, our first study aimed to clarify the pharmacological characteristics of AVL in isolated arteries, in order to explain theconflicts surrounding the use of AVL for treatment of hypertension and to propose thesafe application for this herb.

The World Health Organization (WHO) estimates that over one billion people worldwide suffer from illnesses of the central nervous system [13]. These illnesses not only cause great human suffering, but are also linked with enormous costs-stays in hospital, lost of working hours, early retirement and, in many cases, life-long care. These social, ethical and economic reasons call for the urgent improvement of medical research in this field [135]. Researches have investigated and demonstrated the treatment effect of medicine plants on a number of disorders that occur in brain[14, 110, 118], including the cerebral hemorrhage [49, 52, 79, 119, 121, 123, 143]. Among many kinds of hemorrhage occurring in brain, subarachnoid hemorrhage (SAH) is a type of stroke with high morbidity, mortality, and economic impact. Occurring with an incidence between 2 and 22 per 100,000 persons per year, SAH accounts for about 5% of all strokes, and the modern 30-day mortality is as high as 40%, while about 50% of survivors have permanent disability[29]. Cerebral vasospasm (CVS) is well known as a major complication in SAH patients, afflicts 30% of patients in the aftermath of, and secondary to SAH [45], and research has long been focused on improving the therapies with drugs for preventing CVS after the hemorrhage

[48]. However, the prevention and treatment of this syndrome is difficult, because despite of intensive research for more than half of a century, true nature and cause of vasospasm pathological enigma still remains unclear [32, 103]. Even there has been a number of researches reported the treatment effect of medicinal plants on SAH [19, 34, 83, 120, 122, 123], the investigations of plant effects on cerebral vasospasm is scarce. However, Complementary Chinese herbs have been recently evaluated as systemic drugs for cerebral spasm [3]. Because vasospasm is a significant factor associating pathological conditions of SAH, we believe that medicine plants with treatment on SAH might alsoinhibit this spasm, and our study therefore aimed to investigate this hypothesis. We selected to test the effect of SJ and GB, as they are traditionally believe to have healing effects on many kind of cerebral hemorrhage, including SAH[28, 74, 123], in order to verify if their treatments are partly mediated via the inhibition on vasospasm.

The brain is critically dependent on a continuous and well-regulated blood supply to support its dynamic needs for oxygen and glucose and to remove metabolic by-products of brain activity[92]. Cerebral blood flow (CBF) is highly regulated, involving multiple coordinated mechanisms. Maintaining adequate supply of oxygen and energy to the brain in order to meet the metabolic demands despite the varying neuronal activity and circulatory alterations of everyday life is a key requirement for the regulation of CBF. Most, if not all, brain diseases are associated with changes in CBF, either as cause or a consequence of brain dysfunction. CBF is provided by 2 main pairs of arteries: the vertebral arteries, and the internal carotid arteries. The vertebral arteries arise from the subclavian arteries and unite to form the basilar artery. In addition, this artery is segment of the vasculature where

some of the most important clinical complications of vascular disease occur. Interruption of the blood flow through the basilar artery can lead to severe brain damage, organ malfunction, or death. The unique features of the cerebral circulation make it difficult to extrapolate findings for peripheral blood vessels. The cerebral arteries are more productive and greatly influenced by vasoactive substance than others which make them vulnerable to pathological condition. In the response to a number of pathophysiologic conditions including atherosclerosis, strokes or hypertension, alteration of vascular-regulatory properties of endogenous factor have been reported[15, 23,26]. Basilar artery is an important brain artery, which runs along the ventral aspect of the medulla oblongata and supplies the brain with blood in reptiles, birds and mammals [57]. The responsiveness of this artery seems to reflect changes in cerebral blood flow and local micro - vascular pressure, and study about response of this artery is important because it is one of the major resistance vessels of the brain [57]. In our study materials, we employed porcine basilar artery (PBA) as the specimens because the contraction mechanisms, along with mediating receptors and receptor antagonists of a number of vasoconstrictors had already been previously well identified by our group [58, 85, 87, 88, 89, 90]. In addition, the methods of using organ bath systems for the functional study, the culturing of endothelial cells and the measuring endogenous prostaglandins using immunoassay kits have also been well established in our laboratory [58, 85, 87, 88, 89, 90].

# Chapter 1

## Study 1

## Pharmacological characteristics of *Artemisia vulgaris* L. in isolated porcine basilar artery

1. **ABSTRACT**

In Vietnamese traditional herbalism, there are conflicting opinions about the effect of *Artemisia vulgaris* L. (AVL, English name: mugwort) on hypertension. Some ethnic doctors recommend the use of AVL for treatment of hypertension, whereas others adviseagainst it. The purpose of this study was to clarify the pharmacological characteristics of AVL in isolated arteries to explain theconflicts surrounding the use of AVL for treatment of hypertension. We initially performed a functional study using an organ bath system to investigatethe effect of AVL extract onisolated porcine basilar artery. We then measured the change in intracellular free $Ca^{2+}$ concentration elicited by AVL using cultured smooth muscle cells loaded with the $Ca^{2+}$ indicator fluo-4. Finally, using HPLC, we determined the active components in AVL. AVL induced vasoconstriction at resting tension, and endothelial removal enhanced this effect significantly. Pretreatment with PD123319 (an $AT_2$receptor antagonist), Nω-nitro-L-arginine(a nitric oxide synthase inhibitor), or both, alsoenhanced this effect. AVL-induced contraction was competitively inhibited by methiothepin(a 5-$HT_1$ and 5-$HT_2$receptor antagonist) in the presence of ketanserin (a 5-$HT_2$ receptor antagonist). Removal of extracellular calcium with nifedipine (anL-type $Ca^{2+}$ channel blocker) or ruthenium red (a ryanodine receptor blocker) significantly reduced AVL-induced contraction, whereas losartan (an $AT_1$ receptor antagonist) and diphenhydramine (a $H_1$ receptor antagonist) had no effect on this contraction. AVL increased the intracellular free $Ca^{2+}$ concentration in cultured cells, and this increment was inhibited by methiothepin. HPLC analysis revealed that the retention time of the first peak in the AVL profile was similar to that of the 5-HT standard, and that

addition of 5-HT to the AVL sample enhanced this peak. On the other hand, AVL induced endothelium-independent relaxation under precontractedconditionswith 60 mMKCl. Captopril (an angiotensin converting enzyme inhibitor), atenolol (a $\beta_1$ receptor antagonist) and cimetidine (a $H_2$ receptor antagonist) had no effecton this relaxation. In $Ca^{2+}$-free 60 mMKCl-containing solution, pretreatment with AVL significantly inhibited $CaCl_2$-induced contraction. For the first time, the present study has demonstrated that AVL has two opposite effects, contraction and relaxation, on isolated artery, which may helpto explain the conflicting indications for AVL in traditional herbalism. 5-HT is a significant factor affecting artery contraction in the presence of AVL.

**Keywords:** serotonin, *Artemisia vulgaris* L., mugwort, basilar artery, biphasic effects, hypertension.

## 2. INTRODUCTION

*Artemisia vulgaris* L. (AVL, English name: mugwort, Japanese name: yomogi, Vietnamese name: ngaicuu) has a long history of use in traditional medicine as an agent for treatment of hypertension. AVL is widely used as an alternative medicine for hypertension in the Philippines and has been demonstrated to effectively reverse hypertension in rats [127]. In Vietnam, it has been recommended that AVL be applied alone or included in formulas with other medicinal plants for hypertension [73]. In contrast, some ethnic doctors have advised against the use of AVL for hypertension [55, 63]. To our knowledge, these

variations in advice appear to be based mainly on traditional experience, and there have been no scientific data regarding the vascular response to AVL. Whereas AVL has been reported to exert a relaxant effect in tissues such as mesentery, ileum, jejunum and trachea [62, 95], there have been no reports about AVL-induced contraction. Researchers have suggested that 5-HT pathways might be involved in the pharmacological functions of *Artemisia* plant family [1]. 5-HT has been shown to be present in many edible plants [53] and demonstrated to contribute to contraction effects [33]. A previous study from our laboratory has demonstrated that porcine basilar artery has high sensitivity to 5-HT and shows a sufficient maximal response [85]. In addition, there has been no study investigating effect of AVL on isolated artery. Therefore, the aim of our present study was to clarify the characteristics of the AVL vascular response of porcine basilar artery, in order to explain the current confusion among ethnic doctors regarding the use of AVL for treatment of hypertension.

## 3. MATERIALS AND METHODS

### *3.1. Extract preparation*

Dried leaves of AVL were a gift from Okinawa Chouseiyakusou Co. (Okinawa, Japan). The identity was confirmed by Dr. ThoThi Bui at Department of Veterinary Pharmacology and Internal Medicine, Faculty of Veterinary Medicine, Vietnam National University of Agriculture based on voucher specimen (VDLTY0367) that has been deposited at VuonDuoc Lieu Thu Y Herbarium, Vietnam National University of Agriculture in Vietnam. The AVL dried leaf sample was pulverized to powder with a

coffee blender before being subjected to extraction. The extraction was followed Nakashima *et al*[93] with some modifications. In brief, five grams of the powder was stirred with 100 ml of boiled distilled water for 30 min before filtering through two layers of cheese cloth. The filtrate was centrifuged at 10,000 x *g* for 30 min before being passed through grade No.2 qualitative filter paper (AdvantecMFS Inc., Dublin, CA, USA). The extract was then concentrated at 37ºC using a rotary evaporator at low atmospheric pressure until 10 ml crude extract was obtained from each 5 g of crude powder. Before being tested on tissue, the extract was centrifuged again at 10,000 x *g* for 10 min to remove all of the precipitated substances.

*3.2. Reagents*

We used the following reagents: 2-aminoethyl diphenylborinate (2-APB), 5-hydroxytryptamine (5-HT, serotonin) hydrochloride, acetonitrile, atenolol, bradykinin (BK) acetate salt, captopril, cimetidine, diphenhydramine hydrochloride, Dulbecco′s modified Eagle′ medium, ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), losartan potassium, methanol, nifedipine, Nω-nitro-L-arginine (L-NA), PD123319 ditrifluoroacetate salt, ruthenium red, sodium nitroprusside (SNP), verapamil hydrochloride (Sigma-Aldrich, St, Louis, MO, USA), 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619, Cayman Chemical Co., Ann Arbor, MI, USA), ketanserin tartrate (Kyowa Hakko Kogyo, Tokyo, Japan), and methiothepin maleate (Nippon Roche, Tokyo, Japan). All Krebs salts and other chemicals were general purpose or analytical grade and purchased from NakaraiTes*q*ue (Kyoto, Japan) or Wako (Osaka, Japan). A-10 smooth muscle cells

derived from embryonic rat thoracic aorta were purchased from ATTC (Rockville, MD, USA), calcium kit II fluo-4 was purchased from Dojindo Laboratories (Kumamoto, Japan) and Infinite M200 plate reader was purchased from Tecan (Mannedorf, Switzerland).

*3.3. Functional study*

Basilar arteries were obtained from freshly slaughtered pigs (both sexes, about 6–7 months old, LWD cross-breed) at a local slaughter house and transferred to our laboratory in ice-cold physiological saline solution (PSS, 119 mMNaCl, 4.7 mMKCl, 1.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM $KH_2PO_4$ and 10 mM glucose, pH 7.4) aerated with carbogen (95% (v/v) $O_2$, 5%(v/v) $CO_2$). After the adherent tissues had been carefully removed, several rings approximately 4 mm long were cut from each artery. When re*q*uired, endothelium was removed by gently rubbing the intimal space with a stainless steel rod with a diameter equivalent to the lumen of the artery. Arterial rings were mounted vertically between two L-shaped stainless steel holders, with the upper part fixed to an isometric force transducer (TB-611T, Nihon Kohden Kogyo, Tokyo, Japan), and immersed in a 5-ml water-jacked organ bath containing oxygenated salt solution at 37ºC (pH 7.4). Each suspended ring was left to e*q*uilibrate under a resting tension of 7.5 mN. This tension was chosen because it allowed us to induce maximum contractions in the artery. 60 mMKCl was applied to stimulate the artery. After the contraction has reached the maximum magnitude, artery was washed out, re-e*q*uilibrated and again stimulated with 60 mMKCl. This process was continued until contraction amplitude reached a constant value. The isometric tension was recorded with an amplifier (AP-621G, Nihon Kohden Kogyo,

Tokyo, Japan), digitized with an analog-digital converter (PowerLab/8SP, ADInstruments Co., Castle Hill, NSW, Australia) and stored on the hard disk of a personal computer. The presence of endothelial cells was confirmed pharmacologically by testing the relaxant response to BK under pre-contracted conditions with U46619 (this response is abolished by endothelial denudation; [88]).

*3.3.1. Effect of AVL on resting artery tension*

This experiment attempted to verify AVL-induced contraction. AVL was cumulatively (50 µl to 500 µl/5 ml bath) applied to endothelium-intact or denuded arterial segments under resting tension to obtain the concentration-response curve (CRC). When contraction induced by a concentration of AVL reached the maximal value, the next concentration was applied. Response to 60 mMKCl was taken as 100% and AVL-induced contraction was calculated as a percentage of this response. Because the contraction response of AVL was reproducible on the same arterial segments, pre-treatment with antagonists was performed for 30 min before the next response to AVL was examined. PD123319 (an $AT_2$ receptor antagonist, $10^{-6}$ M) and L-NA (a nitric oxide (NO) synthase inhibitor, $10^{-4}$M) were tested to verify the difference between the responses of endothelium-intact and endothelium-denuded artery. The effect of extracellular $Ca^{2+}$ removal and voltage-dependent L-type $Ca^{2+}$ channel blockade on AVL responses was determined by application of 2 mM EGTA (an extracellular $Ca^{2+}$ chelator) and $10^{-7}$ M nifedipine (an L-type $Ca^{2+}$ channel blocker) . The effect of blockade of histamine $H_1$, $AT_1$, 5-$HT_2$, ryanodine and $IP_3$ receptors on the response to AVL was verified by pre-treatment

with diphenhydramine ($10^{-4}$ M), losartan ($10^{-6}$ M), ketanserin ($10^{-7}$, $3\times10^{-7}$ and$10^{-6}$ *M)*, ruthenium red ($10^{-5}$M) and 2-APB ($10^{-5}$ M). Because there is no selective antagonist for the 5-$HT_1$ receptor, the effect of 5-$HT_1$ receptor antagonism on the AVL response was investigated by testing the effect of methiothepin ($3\times10^{-7}$ and $10^{-6}$ *M*) in the presence of ketanserin ($10^{-6}$ M), following the method reported previously from our laboratory [85]. The log concentration of the $EC_{50}$ value (i.e., the concentration producing a half-maximum response) in the absence or presence of antagonist was calculated. We then established the Schild plot between log (CR-1) and the log (antagonist concentration), in which CR is the ratio of the concentration of AVL producing a 50% maximal response ($EC_{50}$) in the presence of antagonist to $EC_{50}$ in the absence of antagonist. When obtained regression was linear with a slope value close to 1, which indicated the competitive antagonism, we applied the Schild equation to calculate the $pA_2$ value [5].

*3.3.2. Effect of AVL on arteries pre-contracted with 60 mMKCl*:

This experiment attempted to verify that AVL induced relaxation in porcine basilar arteries. KCl was used at 60 mM to induce a steady contraction of arterial segments with the endothelium-intact or denuded, and AVL was added cumulatively (50 µl to 500 µl/5 ml bath) to obtain the CRC. At the end of the experiments, $10^{-4}$ M SNP was added and this relaxation was taken as 100%. AVL-induced relaxation was calculated as a percentage relative to the response elicited by $10^{-4}$ M SNP. After the first response was examined, artery was washed out and re-equilibrated for 30 min before 60 mMKCl was added again to test the 2$^{nd}$ response. Because relaxation response of AVL was reproducible on the same

arterial segments, pre-treatment with antagonists such as captopril (an angiotensin converting enzyme inhibitor, $10^{-6}$ M), atenolol (a selective $\beta_1$ receptor antagonist, $10^{-6}$ M) and cimetidine (a histamine $H_2$ receptor antagonist, $10^{-5}$ M) was performed for 30 min before the next response to AVL was examined. The test for the calcium influx-inhibitive effect of AVL was modified from [62]. Endothelium-denuded arterial segments were allowed to stabilize in normal PSS, which was then replaced with $Ca^{2+}$-free PSS containing EGTA (2 mM) for 30 min in order to remove extracellular $Ca^{2+}$ from the tissues. The solution was finally replaced with $Ca^{2+}$-free and $K^+$-rich (60 mM) PSS. After an incubation period of 30 min, the CRC resulting from addition of extracellular $CaCl_2$ (extracellular $Ca^{2+}$ CRC) to the bath fluid was constructed using the half-logarithmic increment of $Ca^{2+}$ concentration. After the extracellular $Ca^{2+}$ CRCs were found to be super-imposable (after 2 cycles, data not shown), the arterial segment was pre-treated with different concentrations of AVL or verapamil for 30 min, and the next extracellular $Ca^{2+}$ CRC was constructed in the presence of these agents. The concentration-dependent effect of AVL was tested using two concentrations: $0.3\times EC_{50\ (relax)}$ and $EC_{50\ (relax)}$, at which the $EC_{50\ (relax)}$ was the concentration producing a half-maximum response of AVL relaxation (determined from the linear regression obtained between the AVL concentration and relaxation response, equal to 255.5 μl/5 ml bath; data not shown). The change in extracellular $Ca^{2+}$ CRC induced by AVL or verapamil was used to estimate the calcium influx-inhibitory effect.

### *3.4. Measurement of intracellular free $Ca^{2+}$ in cell culture*

A-10 (ATCC® CRL-1476™) smooth muscle cells derived from embryonic rat thoracic aorta were grown in Dulbecco′s modified Eagle′ medium containing 10% fetal bovine serum, 100 unit/ml penicillin and 100 unit/ml streptomycin at 37ºC in a humidified 5% $CO_2$ atmosphere. One day before the experiment, the cells were seeded at a density of $4.5\times10^4$ /cm² in a 96-well plate. For measurement of the change in fluo-4 fluorescence, cells in a 96-well plate were serum-starved for 4 h, and then the calcium indicator fluo-4 was loaded into the cells using Calcium kit II fluo-4 in accordance with the manufacturer′s instructions. In brief, A-10 cells were incubated with 2.5 μM fluo-4AM in the presence of 0.04% pluronic F-127, a dispersing agent to improve the efficiency of loading with fluo-4, and 1.25 mM probenecid, a blocker of organic action transport to prevent leakage of fluo-4 from the cells. After 1 h incubation at 37ºC, the cells were immediately used for measurement of fluo-4 fluorescence at 518 nm emission after excitation at 495 nm using an Infinite M200 plate reader at 37ºC. Emitted fluorescence was measured at 1, 2, 3, 5, and 7 min after addition of AVL or 5-HT to the cells. For tests with the antagonist, pre-treatment with methiothepin was performed for 30 min before treatment with AVL or 5-HT. The concentration-dependent effect of AVL was tested using two concentrations: $0.3\times EC_{50\ (contract)}$ and $EC_{50\ (contract)}$, in which $EC_{50\ (contract)}$ was the concentration producing a half-maximum response in AVL contraction (determined from the linear regression obtained between the AVL concentration and contraction response, equal to 85.9 μl/5 ml bath; data not shown). The ratio of the fluorescence intensity triggered by AVL or 5-HT relative to that of the untreated control was used to estimate the changes in intracellular free $Ca^{2+}$.

*3.5. HPLC analysis of 5-HT*

The method used for HPLC analysis of 5-HT was modified from Hosseinian *et al*[51]. Ten microliters of extract was analyzed using a 150 × 3.0 mm i.d., Luna 5 µm C18 100A column (Waters Corp., Milford, MA, USA). The mobile phases were A: 0.1% acetic acid in double deionized water and B: 0.1% acetic acid in acetonitrile. The gradient conditions were as follows: solvent B: 0 min, 10%; 5 min, 10%; 10-40 min, 40%; 41-50 min, 10%. Other chromatographic conditions were: flow rate: 0.3 ml/min, column temperature: 35ºC and run time, 30 min. Spectroscopic data from all peaks were accumulated in the range 254-600 nm, and chromatograms were recorded at 280 nm. 5-HT in AVL was determined by comparing the retention times of peaks in sample HPLC profile with 5-HT standard. The 5-HT standard was also added to the AVL sample to confirm the presence of 5-HT. The content of 5-HT in AVL was calculated by comparing the sample area (% fluorescence) with that in the standard curve for 5-HT. HPLC analysis of AVL was performed in triplicate.

3.6. *Statistical analysis*

The contraction response was expressed as a percentage of the response obtained with 60 mMKCl. The relaxation response was expressed as a percentage of the response obtained with $10^{-4}$ M SNP. Results are expressed as means ± standard error (S.E.M). The n value represents the number of pigs from which basilar arteries were obtained. Statistical analyses were performed by paired t test or the *Bonferroni* test after one-way analysis of

variance (one-way ANOVA). Significance was established when the probability level was equal to or less than 5%.

## 4. RESULTS

### *4.1. Effect of AVL on resting artery tension*

The contraction effect of AVL on endothelium-intact and denuded porcine basilar artery is shown in Fig 1A. AVL induced strong contraction of porcine basilar artery, with the maximal response exceeding that induced by 60 mMKCl. The contraction was significantly stronger in endothelium-denuded artery. Pre-treatment with PD123319, L-NA, or both, significantly enhanced the AVL-induced contraction of endothelium-intact artery to a similar level (Fig 1B), but did not alter the response of denuded artery (Fig 1C). Because the contraction response to AVL was stronger when the endothelium was removed, we decided to use denuded artery for the tests with antagonists or inhibitors in the subsequent experiments. The effects of extracellular $Ca^{2+}$ removal by 2 mM EGTA and L-type calcium channel blockade by $10^{-7}$ M nifedipine on AVL-induced contraction are shown in Fig 2. AVL contraction was significantly reduced by the two treatments, demonstrating that the response was mediated via extracellular $Ca^{2+}$ mobility, including entry of $Ca^{2+}$ through L-type calcium channels. The effects of ryanodine receptor and $IP_3$ receptor blockade by ruthenium red and 2-APB are shown in Fig 3. AVL contraction was significantly inhibited by pre-treatment with ruthenium red, but was not altered by 2-APB, proving that the contraction was mediated by ryanodine and not by $IP_3$ receptors. In order to verify the involvement of the 5-$HT_2$ receptor, the response was investigated in the

presence of ketanserin and the results are shown in Fig 4A. Ketanserin at $3\times10^{-7}$ M and $10^{-6}$ M significantly shifted the AVL-induced contraction to the right in a concentration-dependent manner, but did not alter the maximal response (Fig 4A). The slope of the Schild plot was 1.19 ± 0.17, which did not differ significantly from unity, and the calculated ketanserin $pA_2$ value was 6.21 ± 0.05 (Fig 4A′). The involvement of the 5-$HT_2$ receptor was investigated by applying methiothepin with $10^{-6}$ M ketanserin pre-treatment to block the 5-$HT_1$ receptor [85], and the results are shown in Fig 4B. In the presence of $10^{-6}$ M ketanserin, methiothepin at $3\times10^{-7}$ M and $10^{-6}$ M significantly and competitively shifted the AVL-induced contraction to the right in a concentration-dependent manner. In addition, methiothepin largely inhibited the contraction induced by low concentrations of AVL but did not alter the maximal response (Fig 4B). The slope of the Schild plot was 1.28 ± 0.35, which did not differ significantly from unity, and the calculated methiothepin $pA_2$ value was 5.94 ± 0.04 (Fig 4B′). In our experiments, $10^{-6}$ M losartan and$10^{-4}$ M diphenhydramine did not cause any change in the AVL-induced contraction (data not shown), demonstrated that this contraction was not mediated via $AT_1$ or histamine $H_1$ receptors.

*4.2. Effect of AVL on artery pre-contracted with 60 mMKCl*

The relaxation effect of AVL on endothelium-intact and endothelium-denuded porcine basilar artery pre-contracted with 60 mMKCl is shown in Fig 5. AVL induced endothelium-independent relaxation. Pre-treatment with AVL induced concentration-dependent inhibition of calcium-induced contraction (Fig 6A), which was similar to the

effect of verapamil (Fig 6B), a typical extracellular $Ca^{2+}$ influx inhibitor. In our experiments, $10^{-6}$ M captopril, $10^{-5}$ M atenolol and $10^{-5}$ M cimetidine did not alter AVL relaxation (data not shown), demonstrating that this relaxation was not mediated by angiotensin converting enzyme, $\beta_1$ receptor or histamine $H_2$ receptors.

*4.3. Effect of AVL on intracellular free $Ca^{2+}$ in cell culture*

AVL and 5-HT induced concentration-dependent increases in the fluorescence ratio of the $Ca^{2+}$ indicator fluo-4 (Fig 7), and methiothepin significantly suppressed these increases (Fig 8).

*4.4. HPLC analysis of 5-HT*

To analyze the 5-HT component in AVL, we first compared the HPLC profile of the AVL extract with that of the 5-HT standard. The representative HPLC profiles of 5-HT and AVL are shown in Fig 9A and Fig 9B. The retention time of the first peak in the AVL profile was similar to that of the standard 5-HT. The presence of 5-HT was further confirmed with the 5-HT standard spiked into the AVL extract, which enhanced the first peak and showed a similar retention time (Fig 9C). We compared the fluorescence area of the first peak in the AVL profile with that of the 5-HT standard curve and thus determined the 5-HT content of AVL to be 41.45 ± 2.29 μg/g dry weight (DW).

## *5.* DISCUSSION

Our present study showed that AVL had two opposite effects, differing according to the conditions of the artery, inducing either contraction or relaxation. AVL-induced contraction of the artery under resting tension, but induced relaxation of the artery pre-contracted with 60 mMKCl. This is the first time that the contraction effect of AVL has been observed. Previous studies investigating the effect of AVL on isolated mesentery, ileum, trachea, and jejunum reported that when applied at the baseline, AVL decreased or did not change the tissues tension [62, 95, 127]. Because the present study represents the first attempt to test AVL on isolated artery, the difference in response can be explained by the difference in the tissues tested previously. As the action on isolated cardiovascular preparations, such as an isolated artery, is usually used for accessing the effect on blood pressure [42], the arterial contraction induced by AVL might result in an increasing of blood pressure, thus partly explaining its contraindication for hypertensive individuals. On the other hand, the relaxant effect of AVL on arterial preparations determined in this study is similar to the results obtained using other isolated tissues [62, 95, 127]. This arterial relaxation induced by AVL contributes to its antihypertensive effect, and therefore partly explains why it has been advocated for treatment of hypertension. A biphasic effect on isolated artery, which includes both of contraction and relaxation, has been observed for several medicinal plants, but vasorelaxation was always stronger than vasocontraction, and therefore would not have interfered with their application for treatment of hypertension [22, 136, 137]. However, our study showed that AVL induced strong contraction of isolated artery, the maximum response exceeding that obtained with 60 mMKCl, suggests that it would be necessary to consider this contraction when evaluating the action of AVL on the

vascular system, and also when applying AVL for treatment of hypertension. The biphasic effect of AVL on isolated artery preparations provides a partial pharmacological explanation for the conflicting opinions regarding its use for treatment of hypertension. Further study is necessary to clarify the conditions for use of AVL in hypertensive individuals, in order to properly exploit its antihypertensive action and to avoid possible adverse effects.

The contraction of porcine basilar artery induced by AVL was significantly attenuated by endothelial cells. Endothelial cells have been known to ameliorate contraction elicited by many agents [68, 109], and on porcine basilar artery, it has been shown that stimulation of $AT_2$ receptors, which are located mainly on endothelial cells, attenuates the contraction via an increase in NO production [86]. The removal of endothelial cells, blockade of $AT_2$ receptors with $10^{-6}$ M PD123319, or the blockade of NO synthase with $10^{-4}$ M L-NA is sufficient to abolish this $AT_2$ -induced attenuation [86]. Similar to these results, AVL contraction was significantly attenuated by the presence of endothelial cells, and this attenuation was decreased by pre-treatment with $10^{-6}$ M PD123319 or $10^{-4}$ M L-NA, thus proving that stimulation of $AT_2$ receptors and NO synthesis had attenuated the contraction. In addition, the presence of $10^{-4}$ M L-NA to $10^{-6}$ M PD123319 did not further increase the contraction enhancement, confirming that activation of $AT_2$ receptors was the main source of NO released in the response [86]. It is difficult to confirm whether $AT_2$-mediated dilation is a cause or a consequence of a reduction in blood pressure [140]. However, in the present study, endothelial cells altered only AVL-induced contraction, but had no influence on its relaxation effect, suggesting that $AT_2$ stimulation only significantly

attenuated AVL-induced contraction, and did not significantly affect its relaxation effect. In addition, losartan (an $AT_1$ receptor antagonist) did not alter AVL-induced contraction, suggesting that AVL has no effect on the $AT_1$ receptor. Selectively agonistic activity of the $AT_2$ receptor has also been observed for other plant-derived compounds, such as compound C21, a well-known naturally occurring $AT_2$ receptor agonist that activates only $AT_2$ while having no effect on the $AT_1$ receptor [134]. Further investigation of the activity of AVL components on ATreceptors might help to determine those that are responsible for this mode of action. Our present data showed that contraction of AVL was explained by the mobility of both extracellular and intracellular $Ca^{2+}$. Functional experiments showed that AVL stimulates and opens L-type calcium channels, leading to entry of extracellular $Ca^{2+}$ into the cells, while also activating the ryanodin receptor to release $Ca^{2+}$ from the sarcoplasmic reticulum. Both of these pathways result in an increment of free intracellular $Ca^{2+}$ in smooth muscle cells and induction of contraction. These results were confirmed by our cell culture study, in which treatment with AVL increased the fluorescence intensity of the calcium indicator fluo-4 in cultured smooth muscle cells, demonstrating that AVL increased the intracellular concentration of free $Ca^{2+}$. The contraction induced by AVL has also been attributed to activation of 5-HT receptors. The previous study in our laboratory has clarified that 5-HT-induced contraction of porcine basilar artery is mediated by two receptor subtypes: 5-$HT_1$ and 5-$HT_2$, both of which are located on artery smooth muscle cells [85]. Using the methods described in that study, involvement of the 5-$HT_2$ receptor in AVL-induced contraction was tested using ketanserin, whereas involvement of the 5-$HT_1$ receptor was tested using methiothepin in the presence of ketanserin[85]. The inhibitory

effect of ketanserin or methiothepin demonstrated the participation of both the 5-$HT_2$ and 5-$HT_1$ receptors in AVL-induced contraction. Because the response to a low concentrations of AVL was largely inhibited by methiothepin, but not by ketanserin, it is likely that the response to low AVL concentrations is predominantly mediated through 5-$HT_1$ receptors, whereas $5HT_2$ receptors participate in the response to high AVL concentrations. These characteristics of the participation of 5-HT receptor subtypes in AVL-induced contraction are similar to those involved in 5-HT contraction [85]. In our cell culture study, methiothepin inhibited the increase in free intracellular $Ca^{2+}$ induced by both AVL and 5-HT on smooth muscle cells, suggesting that, like 5-HT, the increase in free intracellular $Ca^{2+}$ induced by AVL was mediated, at least in part, by the pathways sensitive to methiothepin. Because both the functional and cell culture studies showed that AVL induced contraction via pathways similar to those of 5-HT, we hypothesized that AVL might contain 5-HT as a constituent. 5-HT has been shown to be present in many edible plants, and HPLC is the most widely used method for detecting this component [53]. Using HPLC, we demonstrated that 5-HT was present in AVL at about 41.45 ± 2.29 μg/g DW. Based on this content, the final concentration of 5-HT present in AVL used for the functional study was calculated to be within the range 0.02 - 0.21 μg/ml, being equivalent to $0.95\times10^{-7}$ - $0.95\times10^{-6}$ M 5-HT. This concentration range would be sufficient for 5-HT to induce significant contraction of isolated porcine basilar artery in an organ bath experiment [85], suggesting that 5-HT was responsible, at least in part, for the contraction induced by AVL. The presence of 5-HT as a component of AVL explains its activation of 5-HT receptors. It also partly explains the AVL-induced stimulation of L-type $Ca^{2+}$ channels and

ryanodine receptors, because these pathways are involved in 5-HT-induced arterial contraction [24, 132].However, components other than 5-HT are also likely to participate in AVL-induced contraction. Our functional study showed that the $pA_2$ values of ketanserin and methiothepin for AVL-induced contraction were 6.21 and 5.94, respectively, whereas a previous study conducted in our laboratory, applying the same preparations and methods, obtained $pA_2$ values of 9.58 and 8.92, respectively, for 5-HT contraction [85]. The significantly lower $pA_2$ values for those antagonists on AVL-induced contraction relative to 5-HT contraction suggest that other receptors, which induce contraction via pathways not sensitive to 5-HT receptor antagonists, also participate in the contraction effect.

AVL induces endothelium-independent relaxation of arteries pre-contracted with 60 mMKCl, and this relaxation is mediated, at least in part, by inhibition of calcium influx. Because $K^+$-induced contraction is the result of increased $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels, the relaxation effect on high $K^+$ pre-contracted tissues could be interpreted as antagonism of voltage-dependent $Ca^{2+}$ channels [62]. AVL antagonism of these channels was confirmed by our experiment in which extracellular $Ca^{2+}$ removal (using 2 mM EGTA) suppressed KCl-induced contraction, and pre-treatment with AVL significantly inhibited $Ca^{2+}$-induced contraction of 60 mMKCl-depolarized arteries. Applying the same method, a previous study has reported that AVL inhibited calcium influx in isolated rabbit jejunum, and that this inhibition contributed to the antispasmodic effect of AVL on hyperactive gut, such as that in the treatment of abdominal colic or diarrhea [62]. Our present results are in accordance with that study, and demonstrate that AVL inhibits the calcium channels of isolated arteries, thus contributing to its

antihypertensive effect. However, a previous study [62] and the present one have not yet identified the components responsible for this mode of action. Further studies to identify these components would help to explain the basis of AVL-induced relaxation, not only of arteries but also other tissues such as mesentery, ileum, jejunum and trachea [62, 95, 127].

Our study data suggested that the biphasic effect of AVL on arteries is attributed to its effect on $Ca^{2+}$ channels, because AVL stimulation of those channels was responsible for its contraction effect on resting arteries, whereas AVL inhibition of those channels was responsible for its relaxation of pre-contracted arteries. A biphasic effect on vascular $Ca^{2+}$ channels has also been observed for other plant components, including quercetin and myricetin. While these two plant components have been widely demonstrated to induce vasorelaxation via $Ca^{2+}$ channel inhibition, several recent studies have also shown them to be effective vascular $Ca^{2+}$ channel activators [36, 37, 114]. It is likely that under resting tension, their inhibition of $Ca^{2+}$ channels was overwhelmed by stimulation, but when the $Ca^{2+}$ channels had already been stimulated, antagonistic effects were expressed. Fusi*et al* [36] investigated the biphasic effect of myricetin on $Ca^{2+}$ channels and explained the disappearance of its stimulatory effect under high $K^+$ conditions to limitation of tension development, whereby other $Ca^{2+}$ channel activators could not further stimulate the channels to produce a response greater than that elicited by high $K^+$. Similar to these results, AVL also stimulated $Ca^{2+}$ channels under resting tension and inhibited the channels under high $K^+$ pre-contracted tension. In addition, quercetin, a natural compound with a biphasic effect on $Ca^{2+}$ channels [37, 114], has been identified as a component of AVL [75]. This

suggests that components exerting a biphasic effect on $Ca^{2+}$ channels, such as quercetin, might be present in AVL and contribute to its biphasic effect.

For the first time, our present study has determined that 5-HT is present as a component of AVL. Even 5-HT itself is a potent vasoconstrictor, it has been identified in banana, spinach, strawberry and tomato [80], the plants that are generally recognized to have antihypertensive effects [99, 126, 133, 138, 139]. However, since the measurement of 5-HT in those plants was performed independently to the investigations on their antihypertensive effects, the role of 5-HT in those plants inducing vascular response has not yet evaluated. The amount of 5-HT contained in AVL was determined to be 41.45 μg/g DW, which is higher than that in banana, spinach and strawberry, but about 5 times lower than that in tomato (221.9 μg/g DW) [80]. While the antihypertensive effect of tomato has been confirmed in both animals and humans [99, 139], no study has investigated its effect on isolated artery. Our present study demonstrated that 5-HT in AVL contributes to the plant-induced contraction effect on isolated artery, but has not yet determined the role of 5-HT in the plant-induced *in vivo* vascular effect, which is required to be clarified in future studies. Because 5-HT plays a significant role in AVL-induced contraction, the high sensitivity of porcine basilar artery to 5-HT [85] would partly explain why previous studies of the effects of AVL on other isolated tissues did not detect AVL-induced contraction[62, 95]. 5-HT might also contribute to other pharmacological functions of AVL. Besides its effect on the vascular system, AVL has also been traditionally applied because of its effect on the central nervous system, including analgesic, sedative, anti-depressant and antiepileptic effects [50, 62, 73]. One study has explained the anti-depressant effect of AVL

in terms of brain monoamine oxidase inhibition [76], but no studies have investigated the involvement of 5-HT related mechanisms, which includes many important anti-depressant pathways [142]. It is possible that 5-HT contributes to the anti-depressant effect of AVL, because the neurological activity of many medicinal plants has been attributed to their content of 5-HT and/or 5-HT precursors components, which increase the level of 5-HT in the brain and thus ameliorate psychiatric disorders[107]. Further investigation of the role of 5-HT might also help to explain the antidepressant effect of AVL.

## *6.* CONCLUSION

Our study has revealed a biphasic effect of AVL on isolated artery preparations, which partly explains both its indication and contraindication for treatment of hypertension. This biphasic effect of AVL is mediated, at least in part, by both stimulation and inhibition of $Ca^{2+}$ channels. For the first time, 5-HT has been identified as a component of AVL and shown to contribute to its contraction effect.

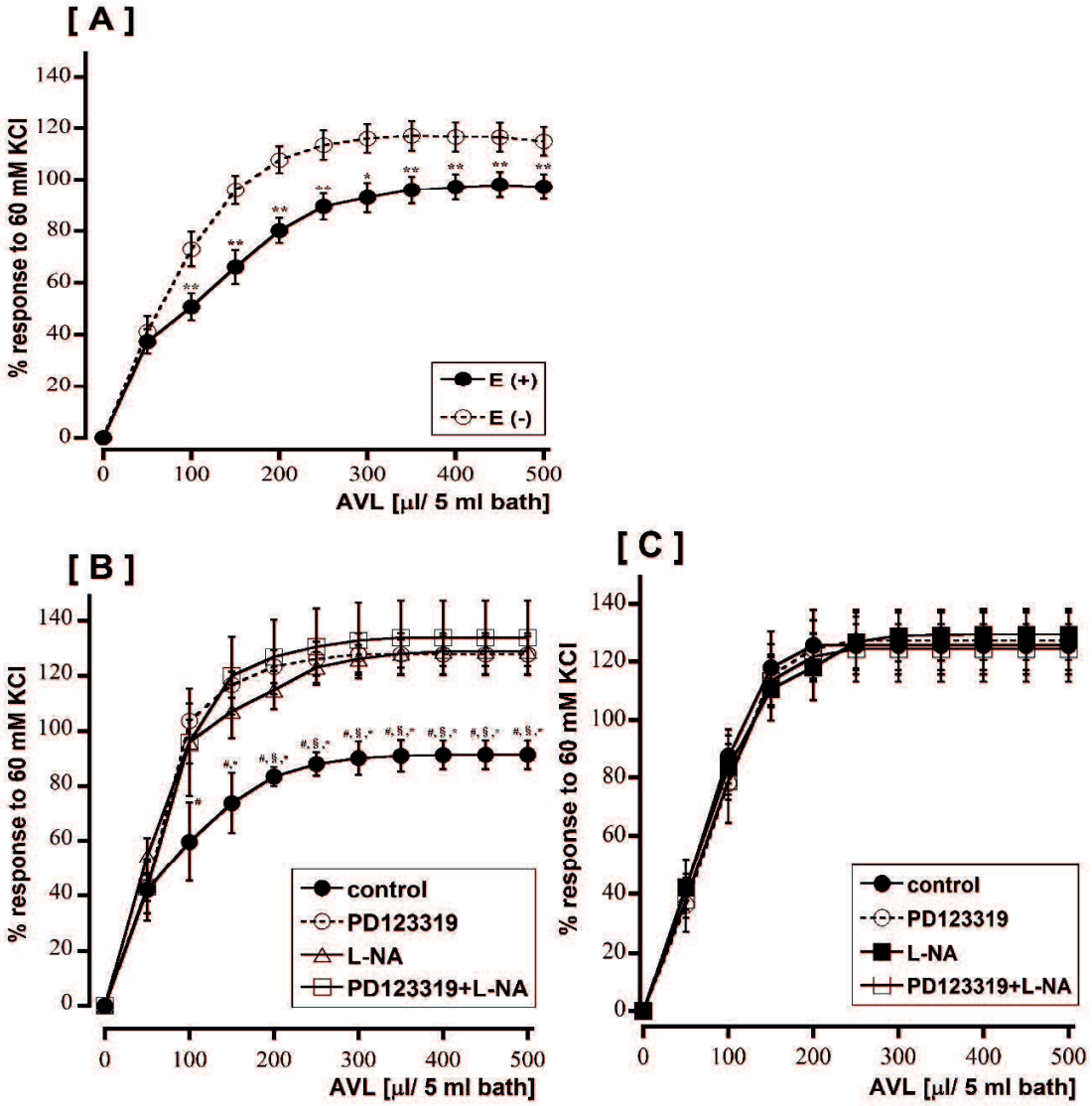


**Fig 1. Contraction effect of AVL on porcine basilar artery. [A] Contraction effect of AVL on endothelial intact and endothelium-denuded artery.** In [A]:●-● on intact artery, ○-○ on denuded artery**.** Each point represents the mean ± S.E.M for 9 different pigs. The responses at each concentration of the intact artery were compared with those of the denuded artery by*paired t* test ($^{*}p < 0.05$, $^{**}p < 0.01$ vs. E (-)). **[B, C] Effect of PD123319, L-NA and PD123319 plus L-NA on contraction effect of AVL on endothelial intact [B] and endothelial denuded [C] artery.** In [B, C]: ●-● in the absence of antagonist, ○-○ in $10^{-6}$ M PD123319, ∆-∆ in $10^{-4}$ M L-NA, □-□ in $10^{-6}$ M PD123319 + $10^{-4}$ M L-NA. Contraction induced by 60 mMKCl (7.96 ± 0.20 mN) was taken as 100%. Each point represents the mean ± S.E.M for 6 different pigs. The responses at each concentration of the extracts in the absence of antagonists were compared with those in the presence of different antagonists by one-way ANOVA followed with *Bonferroni*test ($^{\#}p < 0.05$ vs. PD123319, $^{\S}p < 0.05$ vs. L-NA, $^{*}p < 0.05$ vs. PD123319+L-NA).

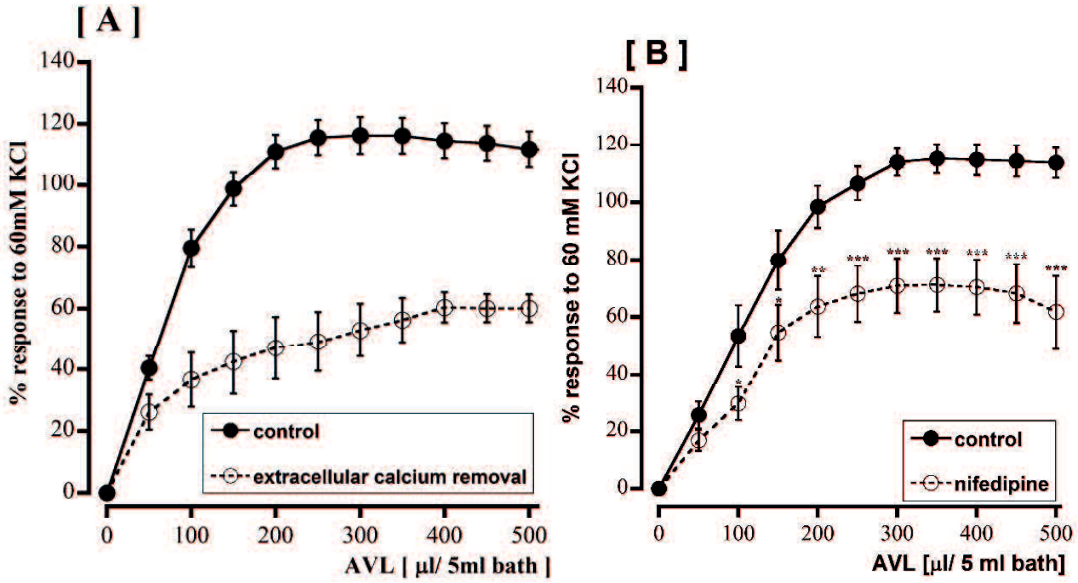


**Fig 2. Effect of extracellular calcium removal and nifedipine on AVL-induced contraction of denuded porcine basilar artery. [A] Effect of extracellular calcium removal.** In [A]:●-● in the presence and ○-○ in the absence of extracellular calcium. **[B] Effect of nifedipine.** In [B]: ●-● in the absence and ○-○ in the presence of $10^{-7}$ M nifedipine. Contraction induced by 60 mMKCl (7.65 ± 0.16 mN) was taken as 100%. Each point represents the mean ± S.E.M for 9 different pigs. The responses at each concentration of the extracts in the presence of antagonist were compared with those of the control by*paired*t test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control).

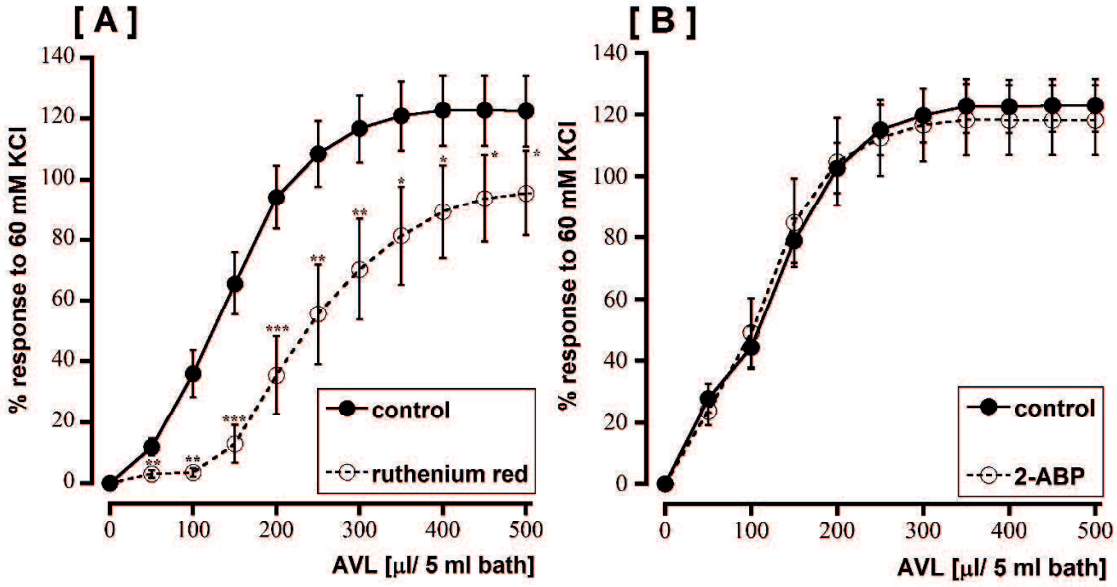


**Fig 3. Effect of ruthenium red and 2-APB on AVL induced contraction of denuded porcine basilar artery. [A] Effect of ruthenium red.** In [A]:●-● in the absence and ○-○ in the presence of $10^{-5}$ M ruthenium red. **[B] Effect of 2-APB.** In [B]: ●-● in the absence and ○-○ in the presence of $10^{-5}$ M 2-APB. Contraction induced by 60 mMKCl (7.69 ± 0.70 mN) was taken as 100%. Each point represents the mean ± S.E.M for 7 different pigs. The responses at each concentration of the extracts in the presence of antagonist were compared with those of the control by*paired*t test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control).

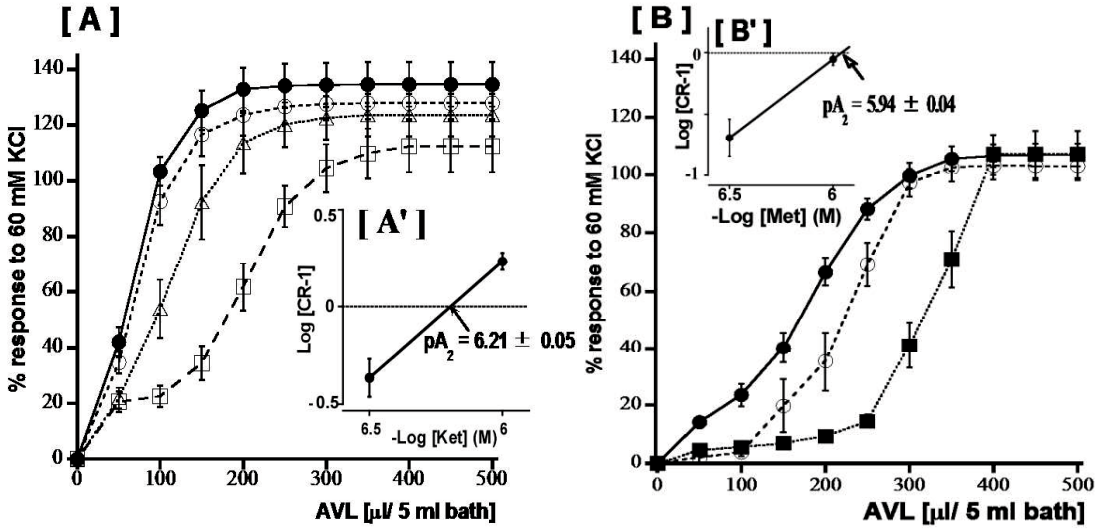


**Fig 4. Effect of 5-HT receptors antagonists on AVL-induced contraction of denuded porcine basilar artery. [A] Effects of ketanserin.** In [A]: ●-● in the absence and presence of increasing concentrations of ketanserin: ○-○ in $10^{-7}$ M ketanserin, ∆-∆ in $3\times10^{-7}$ M ketanserin and □-□ in $10^{-6}$ M ketanserin. **[A′] Schild plot showing effect of ketanserin on AVL-induced contraction**. **[B] Effects of methiothepin in the presence of $10^{-6}$ M ketanserin.** In [B]: ●-● in the absence and presence of increasing concentrations of methiothepin: ○-○ in $3\times10^{-7}$ M methiothepin, ∆-∆ in $10^{-6}$ M methiothepin. **[B′] Schild plot showing effect of methiothepin on AVL-induced contraction in the presence of ketanserin**. CR: an equi-effective concentration-ratio of AVL, i.e., the ratio of the concentration of agonist producing a 50% maximal response ($EC_{50}$) in the presence of antagonist to $EC_{50}$ in the absence of antagonist. Contraction induced by 60 mMKCl (7.60 ± 0.09 mN) was taken as 100%. Each point represents the mean ± S.E.M for 6 different pigs.

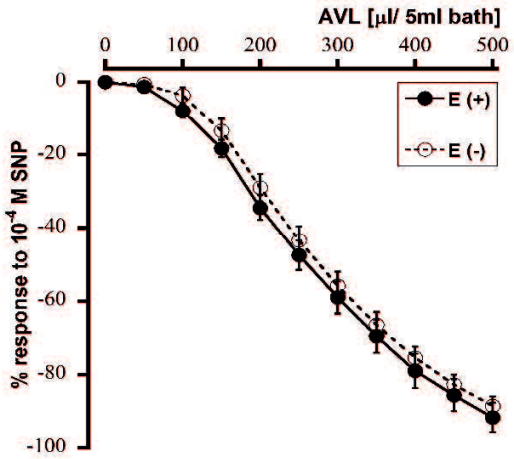


**Fig 5. Relaxation effect of AVL on porcine basilar artery pre-contracted with 60 mMKCl.** ●-● on intact artery, ○-○ on denuded artery**.** Relaxation induced by $10^{-4}$ M SNP (7.56 ± 0.29 mN) was taken as 100%. Each point represents the mean ± S.E.M for 9 different pigs.

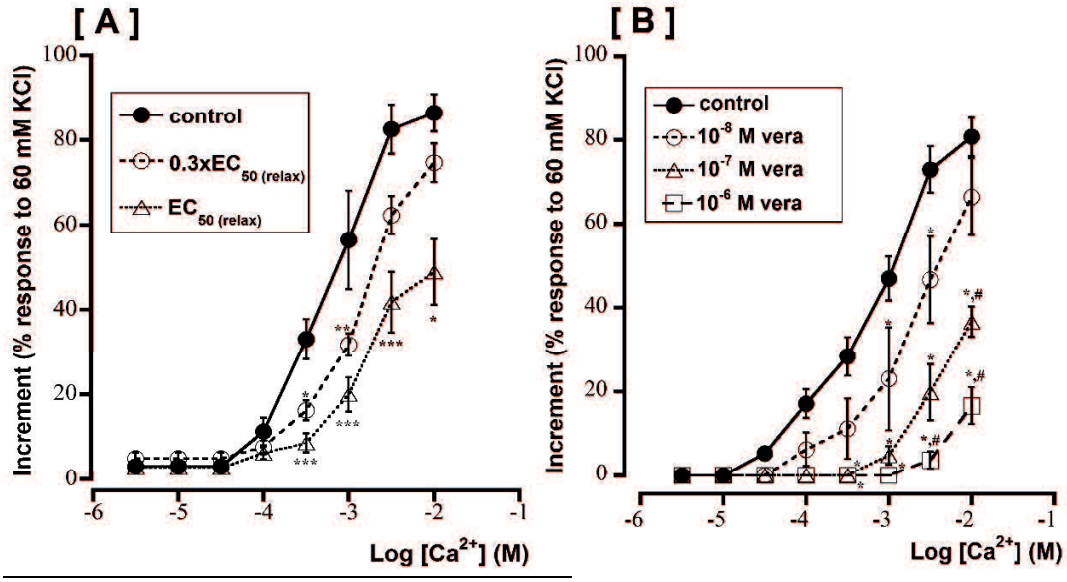


**Fig 6. Effect of AVL and verapamil on the $Ca^{2+}$induced contractionof artery depolarized with 60 mMKCl in calcium-free medium.[A] Effect of AVL**. In [A]: in the absence: ●-● and in the presence of increasing concentrations of AVL: ○-○ in $0.3\times EC_{50\ (relax)}$ AVL and ∆-∆ in $EC_{50\ (relax)}$ AVL. Each point represents the mean ± S.E.M for 6 different pigs. The responses at each concentration in the absence of extract were compared with those in the presence of extract at different concentrations by one way ANOVA followed by *Bonferroni*test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control). **[B] Effect of verapamil.** In [B]: in the absence: ●-● and in the presence of increasing concentrations of verapamil: ○-○ in $10^{-8}$ M verapamil, ∆-∆ in $10^{-7}$ M verapamil and □-□ in $10^{-6}$ M verapamil. Contraction induced by 60 mMKCl (7.60 ± 0.12 mN) was taken as 100%. Each point represents the mean ± S.E.M for 6 different pigs. The responses at each concentration in the absence of verapamil were compared with those in the presence of verapamil at different concentrations by one-way ANOVA followed by *Bonferroni*test ($^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. $10^{-8}$ M vera).

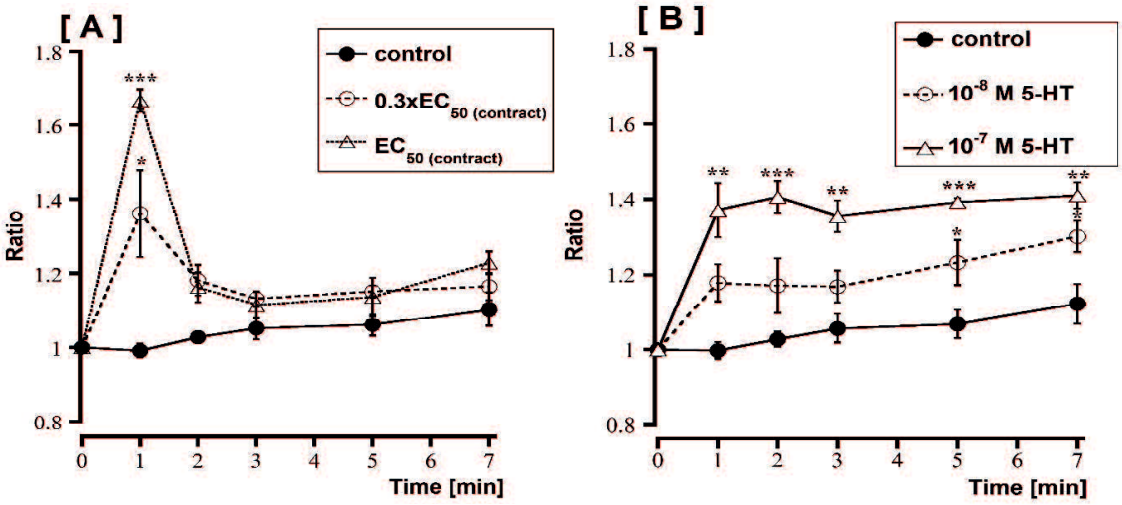


**Fig 7. Change in fluorescence ratio of the $Ca^{2+}$ indicator fluo-4 induced by treatment of cells with AVL or 5-HT**. **[A] Change in the fluorescence ratio of fluo-4 induced by AVL**. In [A]: ratio changes in the absence: ●-● and in the presence of increasing concentrations of AVL: ○-○ in $0.3\times EC_{50\ (contract)}$ AVL and ∆-∆ in $EC_{50\ (contract)}$ AVL. **[B] Change in the fluorescence ratio of fluo-4 induced by 5-HT.** In [B]: ratio changes in the absence: ●-● and in the presence of increasing concentrations of 5-HT: ○-○ in $10^{-8}$ M 5-HT and ∆-∆ in $10^{-7}$ M 5-HT. The responses at each time point in the untreated control were compared with those of AVL or 5-HT treatment at different concentrations by one-way ANOVA followed with *Bonferroni*test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control). Each point represents the mean ± S.E.M for 4 tests.

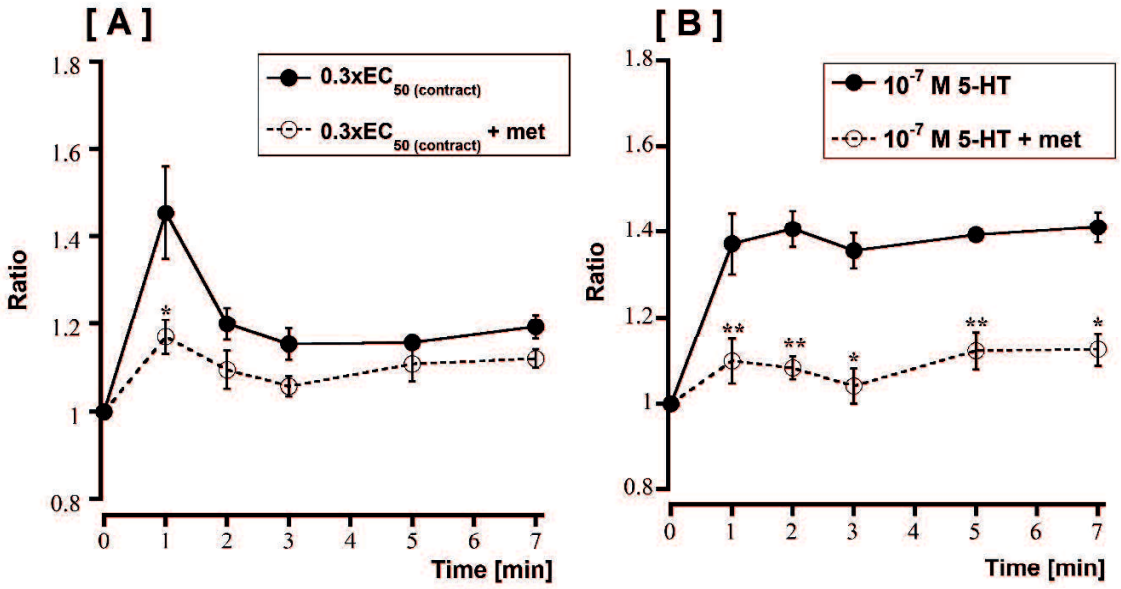


**Fig 8. Effect of methiothepin on the change in fluorescence ratio of $Ca^{2+}$ indicator fluo-4 induced by treatment of cells with of AVL or 5-HT**. **[A] Effect of methiothepin on change in fluorescence ratio of fluo-4 induced by AVL**. In [A]: ratio changes induced by $0.3\times EC_{50\ (contract)}$ AVL in the absence: ●-● and in the presence: ○-○ of $10^{-6}$ M methiothepin. **[B] Effect of methiothepin on change in fluorescence ratio of fluo-4 induced by 5-HT.** In [B]: ratio changes induced by $10^{-7}$ M 5-HT in the absence: ●-● and in the presence: ○-○ of $10^{-6}$ M methiothepin. The responses at each time point induced by AVL or 5-HT in the absence and in the presence of methiothepin were compared by *paired* t test ($^{*}p < 0.05$, $^{**}p < 0.01$). Each point represents the mean ± S.E.M for 4 tests.

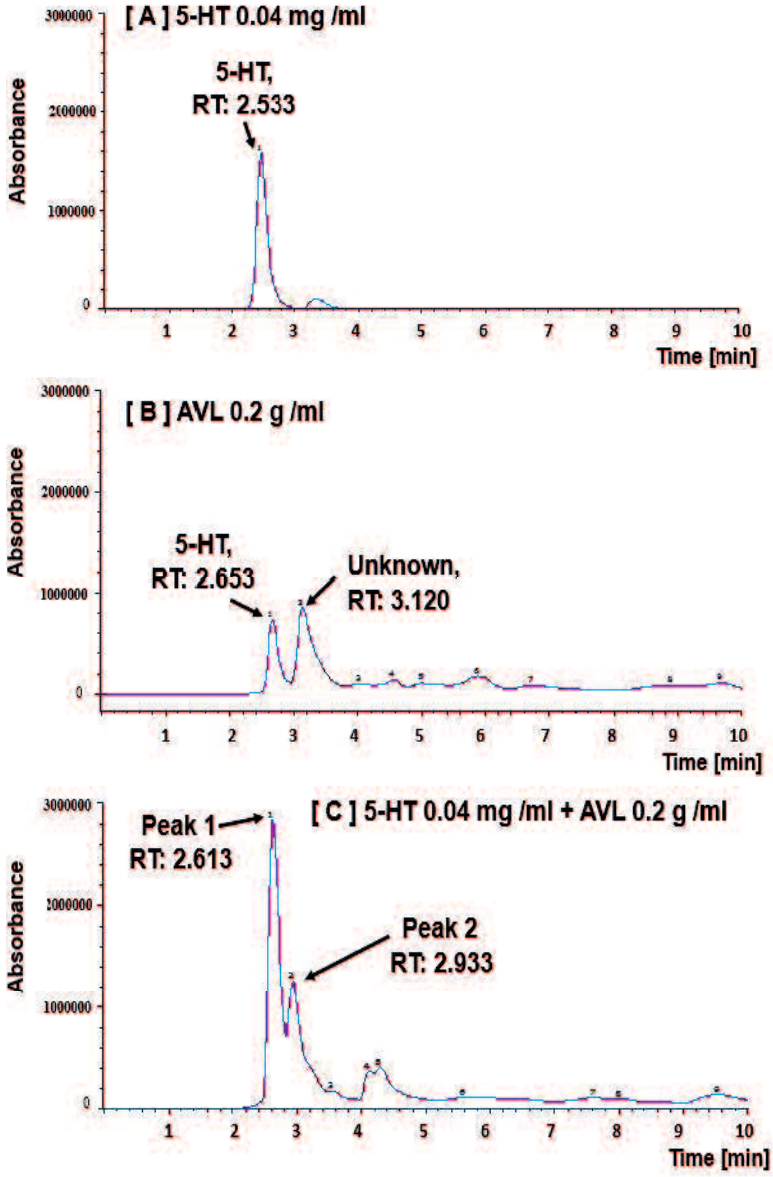


**Fig 9. Representative HPLC chromatograms of [A] 5-HT standard (0.04 mg/ml), [B] AVL (0.2 g/ml) and [C] the 5-HT standard (0.04 mg/ml) used to spike the AVL sample (0.2 g/ml).**

# Chapter 2

# Study 2

**Antagonistic effects of *Gingko biloba* leaf and *Sophora japonica* L. flower bud extracts on cerebral vasoconstriction in response to histamine, 5-hydroxytryptamine, U46619 and bradykinin**

## 1. ABSTRACT.

The aim of this study was to evaluate, for the first time, the antagonistic effects of *Gingko biloba* leaf (GB) and *Sophora japonica* L. flower bud (SJ) extracts on cerebral vasoconstriction in response to KCl, extracellular $Ca^{2+}$, histamine, 5-hydroxytryptamine (5-HT), 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin (PG) $F_{2\alpha}$ (U46619) and bradykinin (BK), in order to explain their traditional application for diseases associated with cerebral vasospasm. Isolated porcine basilar artery (PBA) and endothelial cells from them were used as the study materials. Neither SJ nor GB had any effect on the contractions induced by KCl and extracellular $Ca^{2+}$. SJ significantly inhibited the contraction induced by histamine, 5-HT, U46619 and BK, whereas GB inhibited histamine-induced contraction, but had no effects on the contractions induced by 5-HT, U46619 and BK. In the presence of diphenhydramine (a $H_1$ receptor antagonist), ketanserin (a 5-$HT_2$ receptor antagonist) and ONO-3708 (a thromboxane (TX) $A_2$/PGreceptor antagonist), the inhibitory effects of these extracts on the contractions induced by histamine, 5-HT and U46619 were abolished. SJ significantly inhibited the contractions induced by BK and $PGF_{2\alpha}$, but in the presence of ONO-3708 ($10^{-5}$ M) had no effect on them. BK enhanced the production of $PGF_{2\alpha}$from cultured PBA endothelium cells, and SJ significantly attenuated this enhancement. These results suggest that SJ and GB have $H_1$-antagonistic effect, and that SJ also attenuate cerebral vasoconstriction mediated via 5-$HT_2$ and $TXA_2$/PG receptors. These findings appear to explain why SJ has been used traditionally as a therapeutic medication for cerebral vasospasm after cerebral hemorrhage.

**Keywords:** Cerebral spasm, endothelial cell, *Gingko biloba*, porcine basilar artery, receptors, *Sophora japonica* L.,vasocontraction.

## 2. INTRODUCTION

Vasospasm can be seen in patients with subarachnoid hemorrhage (SAH), intraventricular hemorrhage, subdural hematoma, contusions [84], intracerebral hemorrhage [104] and head injury [100]. Cerebral vasospasm is consistently the leading cause of poor outcome and death after SAH, adversely affecting more than 1 in 5 of all patients who have suffered SAH and survived [30]. Cerebral vasospasm is a devastating condition in which intracranial arteries constrict and diminish blood flow to regions of the brain supplied by the affected arteries. The precise pathogenic mechanisms responsible for cerebral vasospasm remain obscure, and effective therapies remain elusive [60]. Among various medicinal therapies currently being used or investigated, only a few have been proven to be useful for amelioration of cerebral vasospasm. Complementary Chinese herbs have been recently evaluated as systemic drugs for cerebral spasm [3].A number of medicinal plants have been reported to exert antispasmodic effects via antagonism of spasmogen receptors in isolated mesenteric, ileum, trachea and jejunum, explaining their treatment in hyperactive gut and airways disorders [62, 95]. However, no similar studies have tested the receptor antagonism of medicinal plants on isolated cerebral arteries with a view to potential application for treatment of cerebral vasospasm. In the present study we investigated the effects of *Ginkgo biloba* leaf (GB) and *Sophora japonica* L. flower bud (SJ) extracts, because both herbs have been used traditionally for treatment of many cerebral disorders

[28, 74]. GB has been widely reported to have a protective effect against SAH [123]. Similarly, in Vietnamese ethnic medicine, SJ has been recorded as a remedy for cerebral hemorrhage [28, 74]. Here we investigated the anti-spasm effects of SJ and GB on cerebral artery to acquire information that would help to explain their usage for treatment of cerebral disorders associated with spasm, such as SAH and other forms of cerebral hemorrhage. For this purpose, we used specimens of basilar artery, which has been widely reported to be affected by cerebral vasospasm, and highly associated with poor outcome after SAH [124].First, we investigated the effect of SJ and GB extracts on arterial contraction induced by KCl and extracellular $Ca^{2+}$ to clarify whether they inhibit calcium influx, as this mode of action has been reported to mediate the relaxation of isolated arteries by a number of medicinal plants [96]. We then examined the effects of SJ and GB on arterial contraction induced by histamine, 5-hydroxytryptamine (5-HT, serotonin), U46619(a thromboxane (TX) $A_2$/prostaglandin (PG) $H_2$ receptors agonist) and bradykinin (BK), which have all been implicated in cerebral vasospasm [81, 116]. The study material we employed was porcine basilar artery (PBA) as the contraction mechanisms, mediating receptors and receptor antagonists of those vasoconstrictors had already been well established for PBA by our group [58, 85, 87, 88, 89, 90]. We aimed to clarify whether SJ and GB exert antagonistic effects on receptors mediating the vasoconstriction of basilar artery, in order to explain the anti-spasm effects of these medicinal plants.

## 3. MATERIALS AND METHODS

*3.1.Plant collection and preparation of crude extracts*

The SJ sample was collected from the traditional medicine plant garden at the *Department of Internal Medicine and Pharmacology*, Faculty of Veterinary Medicine, Hanoi National University of Agriculture, Vietnam. The GB sample was purchased from KawamotoyaShouten Company (Yokohama, Japan). The identities of GB and SJ were confirmed by Dr. ThoThi Bui at Department of Veterinary Pharmacology and Internal Medicine, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, based on voucher specimens that had been deposited at VuonDuoc Lieu Thu Y Herbarium, Vietnam National University of Agriculture. Both plant samples were pulverized to powder in a coffee blender before being extracted using the procedure reported by Nguyen *et al.*[96]. In brief, 5 g of each powder was stirred with 100 ml of boiled distilled water for 30 min, and then filtered through two layers of cheese cloth. The filtrates were centrifuged at 10,000 x*g* at 4ºC for 30 min before passage through grade No.2 qualitative filter paper (*Advantec*MFS Inc., *Dublin, CA, USA*). The extracts were later concentrated at 37ºC using a rotary evaporator at low atmospheric pressure until 10 ml of crude extract had been obtained from 5 g of each crude powder. Before tested to the tissues, extracts were centrifuged again at 10,000 x*g* for 10 min to remove all of the precipitated substances. For our investigation, we used three different concentrations of SJ and GB, i.e. 25 µl, 50 µl and 100 µl of extracts per 5 ml of organ bath fluid, which were abbreviated as SJ-25, SJ-50, SJ-100 and GB-25, GB-50, GB-100, respectively.

*3.2.Isolated tissue preparation*

Basilar arteries were obtained from freshly slaughtered pigs (both sexes, about 6–7 months old, LWD cross-breed) at a local slaughterhouse and transferred to our laboratory in ice-cold physiological saline solution (PSS, 119 mMNaCl, 4.7 mMKCl, 1.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM $KH_2PO_4$ and 10 mM glucose, pH 7.4) aerated with carbogen (95% (v/v) $O_2$, 5% (v/v) $CO_2$). Each artery was dissected free of adherent tissues. Several rings, each approximately 4 mm long were cut from each artery. The rings were mounted vertically between two L-shaped stainless steel holders with the upper part fixed to an isometric force transducer (TB-611T, Nihon Kohden Kogyo, Tokyo, Japan), and immersed in a 5-ml water-jacketed organ bath containing oxygenated salt solution at 37ºC (pH 7.4). Each suspended ring was left to equilibrate for 120 min under a resting tension of 7.5 mN. This tension was chosen because it allows maximum contractions to be induced in the PBA. We applied 60 mMKCl every 30 min until the contraction amplitude reached a constant value, and this value was used as 100% contraction [58, 91]. Changes in the KCl concentration of the physiological saline were compensated by equimolar adjustment of the NaCl concentration. The isometric tension was recorded with an amplifier (AP-621G, Nihon Kohden Kogyo, Tokyo, Japan), digitized with an analogue-digital converted (PowerLab/8SP, ADInstruments Co., Castle Hill, NSW, Australia) and stored on the hard disk of a personal computer. When re*q*uired, the endothelium was removed by gently rubbing the intimal space with a stainless steel rod with a diameter equivalent to that of the arterial lumen. The presence of endothelial cells was confirmedpharmacologically by testing the relaxant followed by the contraction response to BK under pre-contracted

conditions with U46619 (this response is abolished by endothelium denudation [88]), and the absence of endothelial cells on denuded segments was confirmed by the absence of this response.

*3.3.Reagents*

We used the following reagents: papaverine hydrochloride from NacalaiTes*q*ue (Kyoto, Japan), histamine dihydrochloride, 5-HT hydrochloride, $PGF_{2\alpha}$,BK acetate salt, uridine 5′-triphosphate (UTP), cimetidine, diphenhydramine hydrochloride, ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) (Sigma-Aldrich Co., St. Louis, MO, USA), U46619 (Cayman Chemical Company, Ann Arbor, MI), ketanserin tartrate (Kyowa Hakko Kogyo, Tokyo), Nω-nitro-L-arginine (L-NA) (Aldrich, Milwaukee, WI, USA), the L-arginine salt of 9,11-dimethylmethano-11,12-methano-13,14-dihydro-13-aza-14-oxo-15-(β)-cyclopentyl-ω-pentanor-$TXA_2$ (ONO-3708) (Ono Pharmaceutical Co, Osaka, Japan), HOE140 (Peptide Institude, Inc. Osaka, Japan) and $PGF_{2\alpha}$ EIA kit (Cayman Chemical Co.). All Krebs salts and other chemicals were general purpose or analytical grade and purchased from NacalaiTes*q*ue (Kyoto, Japan) or Wako (Osaka, Japan).

*3.4.Effect of plant extracts on arterial contraction induced by KCL and extracellular* $Ca^{2+}$

The concentration response curve (CRC) of KCl for endothelium-denuded artery was constructed using logarithmic increments in KCl concentration. Because this CRC was virtually reproducible after the first response, we investigated the effect of extracts on KCl-

induced contraction by adding them directly to the bath fluid for 30 min before constructing the second CRC. The effects of the plant extracts on PBA CRCs for extracellular $Ca^{2+}$ were investigated using the method reported previously by our group [96]. In brief, endothelium-denuded arterial segments were allowed to stabilize in normal PSS, which was then replaced with $Ca^{2+}$-free PSS containing EGTA (2 mM) for 30 min in order to remove extracellular $Ca^{2+}$ from the tissues. The solution was finally replaced with $Ca^{2+}$-free and $K^{+}$-rich (60 mM) PSS. After an incubation period of 30 min, the CRC resulting from addition of $CaCl_2$ (extracellular $Ca^{2+}$ CRC) to the bath fluid was constructed using the half-logarithmic increment of $Ca^{2+}$ concentration. After the extracellular $Ca^{2+}$ CRCs were found to be super-imposable (after 2 cycles, data not shown), the arterial segment was treated with plant extracts for 30 min, and the next extracellular $Ca^{2+}$ CRC was constructed in the presence of these agents. The change in extracellular $Ca^{2+}$ CRC induced by these extracts was then used to estimate the calcium influx-inhibitory effects.

*3.5.Effects of plant extracts on contraction induced by receptor-mediated vasoconstrictors*

Because receptors mediating the contraction of PBA in response to histamine, 5-HT and U46619 have been identified on smooth muscle cells, the effects of plant extracts on this type of contraction were tested using denuded artery, in order to abolish any non-specific modulation effect of endothelial cells. The contraction in response to histamine was tested in the presence of $10^{-4}$ M cimetidine to eliminate any effect of the relaxant response [87]. Because BK induces contraction of only endothelium-intact PBA via the main endothelium-derived contracting factor $PGH_2$ [88], which is then converted to the active

vasoconstrictor $PGF_{2\alpha}$[58], the effects of plant extracts on contraction induced by BK and $PGF_{2\alpha}$ were investigated using endothelium-intact artery.

*3.6.Effects of plant extracts on contraction induced by histamine, 5-HT and U46619*

The CRC of each agonist was constructed using logarithmic or half-logarithmic increments in agonist concentration. After the first CRC ($CRC_1$) had been constructed, the segment was washed and equilibrated to restore the resting tension before constructing the second CRC ($CRC_2$). Depending on the agonist, a similar process was repeated to obtain a number of CRCs to determine that from which CRC the response would become superimposable. These tests showed that the $CRC_2$ for histamine and U46619, and the $CRC_3$ for 5-HT, were superimposable (data not shown). Based on these results, when the contraction response had become reproducible, we investigated the effects of plant extracts by adding them directly to the bath fluid for 30 min before examining the next response. The change in the CRC induced by this pre-treatment was used to evaluate the effects of the extracts.

*3.7. Effects of plant extracts on contraction induced by BK and* $PGF_{2\alpha}$

BK has been reported to induce relaxation followed by contraction on PBA, and we investigated this biphasic response by employing the same method as that used in a previous study from our laboratory [88]. UTP was used to produce stable contractions corresponding to approximately 40% of the maximal contraction elicited by 60 mMKCl, and then BK was applied to induce relaxation followed by contraction. The difference

between the relaxation induced by $10^{-6}$M BK and the following contraction was taken as the BK-induced contraction. At the end of experiment, papaverine (100 μM) was applied to produce maximum relaxation. The difference between the UTP pre-contraction and papaverine-induced relaxation was taken as 100% to calculate the BK-induced relaxation [88]. Because the biphasic response of PBA to BK was infrequent and not reproducible on the same segment, effects of plant extracts were investigated by applying them to segments from the same artery with a control to avoid tachyphylaxis which might be produced by repeating application of BK on the same preparation [88]. When inhibition of BK-induced contraction was observed, the extracts were examined again under conditions where $10^{-4}$M L-NA (a NO synthase inhibitor) was applied to abrogate the relaxation [89]. We constructed the CRC for $PGF_{2\alpha}$ by using logarithmic increments of $PGF_{2\alpha}$ concentration. Because this CRC was infrequent and not reproducible on the same segment, we examined the effects of plant extracts by applying them to segments from the same artery with a control.

### *3.8.Effects of plant extracts on agonist-induced contraction in the presence of receptor antagonists*

When a plant extract at a certain concentration inhibited the contraction induced by an agonist but did not alter its maximum response, we examined the effect again under conditions where antagonists were applied to block receptor activation. Antagonists, including $10^{-5}$ M diphenhydramine, $10^{-6}$ M ketanserin and $10^{-5}$ M ONO-3708, were applied

to fully block activation of the $H_1$ receptor [87], 5-$HT_2$ receptor [85] and $TXA_2$/PG receptor [88, 90] on PBA. Because agonist CRCs were reproducible under these conditions, the effects of the extracts were tested by adding them to the bath fluid for 30 min before examining the next response. These experiments were performed to determine the effects of receptor blockade on inhibition induced by the plant extracts.

### *3.9. Effects of plant extracts on BK-enhanced $PGF_{2\alpha}$ production by cultured PBA endothelial cells*

Culture of PBA endothelial cells (PBAECs) was performed using methods reported previously by our group [91]. Primary PBAECs were isolated by infusing 0.05% trypsin-EDTA solution into the vessel through a polyethylene tube (SP 10, ID 0.28 mm, OD 0.61 mm, Natsume, Tokyo, Japan) and cultured in a growth medium containing 45% DMEM, 45% nutrient mixture F-12 HAM, 10% horse serum, and an antibiotic mixture comprising 100 units/ml penicillin, 100 µl/ml streptomycin, and 2.5 µl/ml amphotericin B. Endothelial cells were characterized by their morphology using phase-contrast microscopy for fluorescent acetylated low-density lipoprotein [91]. Endothelial cells that had been cultured for less than six passages were used. Quantification of $PGF_{2\alpha}$production enhanced by BK treatment on PBAEC culture medium was performed using a method that had been reported previously by our group [58]. When $PGF_{2\alpha}$production was measured, a mixed medium containing 50% DMEM and 50% F-12 HAM without phenol red was used to avoid interference with the fluorometric assay [91]. Because this study involved application of

plant extracts to culture medium, cells were serum-starved by being cultured without serum for another 2.5 h to eliminate any influence of residual serum. Confluent endothelial cells (3-5 x$10^{-6}$) were then treated with or without plant extracts for 30 mins before exposure to BK ($10^{-7}$ M) for 1 h. The concentration of $PGF_{2\alpha}$ in the PBAEC culture medium supernatant was measured using a $PGF_{2\alpha}$ enzyme immunoassay kit (Cayman Chemical Co.) in accordance with the instructions provided by the manufacturer. The data were analyzed using a computer spreadsheet provided on the manufacturer's website. The sampling times for quantifying the $PGF_{2\alpha}$were set to 0 and 60 min after changing the medium [58].

*3.10. Data measurement and analysis*

The contraction response was expressed as a percentage of the response obtained with 60 mMKCl. The relaxation response was expressed as a percentage of the response obtained with 100 μM papaverine. Results are expressed as the means ± standard error (SEM). The n value represents the number of pigs from which basilar arteries were taken. Statistical analyses were performed by Student's *t*test or the *Bonferroni* test after one-way analysis of variance. Receptor antagonistic effects were evaluated by comparing the maximum response, -log$EC_{50}$ (-log molar concentration needed to produce 50% of the maximum response), and dose-ratio, following the method used in a previous study [95]. Dose-ratio was calculated by dividing the $EC_{50}$ value of the agonist in the absence of extract by the value in the presence of extract, in order to estimate the change in agonist concentration

required to obtain the same response. Significance was established when the probability level was equal to or less than 5%.

## 4. RESULTS

### *4.1. Effects of plant extracts on contraction induced by KCl and extracellular $Ca^{2+}$*

Neither SJ nor GB altered the CRCs induced by KCl or extracellular $Ca^{2+}$ (Fig. 1A, Fig. 1B, Fig. 1C).

### *4.2. Effects of plant extracts on histamine-induced contraction*

The effects of pre-treatment with SJ and GB extracts on histamine-induced contraction are shown in Fig. 2A and 2B. These figures indicate that SJ and GB dose-dependently inhibited the contraction induced by histamine. SJ-25 and GB-50 significantly shifted the CRC of histamine to the right without suppression on the maximum contraction. When plotted as percentage of the original maximum response, a rightward displacement effect of SJ-25 and GB-50 on the histamine-induced CRC was evident with a significant reduction of the $-\log EC_{50}$ (from 4.42 ± 0.09 in the control to 2.74 ± 0.05 with SJ-25 and 3.36 ± 0.11 with GB-50), corresponding to dose-ratios of 35.21 ± 12.86 and 8.59 ± 2.51, respectively (Table 1). The inhibitory effects of SJ-25 and GB-50 were abolished in the presence of the $H_1$ receptor antagonist, $10^{-5}$M diphenhydramine (Fig. 2C). The $-\log EC_{50}$ values were not different from those of the control (2.49 ± 0.12 with SJ-25 and 2.48 ± 0.03

with GB-50 vs. 2.49 ± 0.04 for the control), and the dose-ratio were reduced to 1.07 ± 0.12 and 1.01 ± 0.03, respectively (Table 1).

### ***4.3.*** *Effect of plant extracts on 5-HT-induced contraction*

The effects of pre-treatment with SJ and GB extracts on 5-HT-induced contraction are shown in Fig. 3A and 3B. Fig. 3A shows that SJ significantly inhibited the contraction of 5-HT in a dose-dependent manner, while GB had no effect on this agonistic contraction (Fig. 3B). SJ-25 significantly shifted the CRC of 5-HT to the right without suppression of the maximum contraction. When plotted as a percentage of the original maximum response, rightward displacement of the 5-HT-induced CRC by SJ-25 was evident with a significant reduction of the -log $EC_{50}$ value, from 6.98 ± 0.15 for the control to 5.48 ± 0.21, corresponding to a dose-ratio of 85.98 ± 3.54 (Table 1). The inhibitory effect of SJ-25 was abolished in the presence of the 5-$HT_2$ receptor antagonist, $10^{-6}$ M ketanserin (Fig. 3C). The -log$EC_{50}$ value was similar to that of the control (6.07 ± 0.22 vs. 6.14 ± 0.18), corresponding to a dose-ratio of 1.21 ± 0.15 (Table 1).

### ***4.4.*** *Effects of plant extracts on U46619-induced contraction*

The effects of pre-treatment with SJ and GB extracts on U46619-induced contraction are shown in Fig. 4A and 4B. Fig. 4A shows that SJ significantly and dose-dependently inhibited the contraction induced by U46619 whereas GB had no effect on this contraction (Fig. 4B). SJ-50 and SJ-100 significantly shifted the CRC of U46619 to the

right without suppression of the maximum response. When plotted as a percentage of the original maximum response, rightward displacements of the U46619-induced CRC by SJ-50 and SJ-100 were evident with a significant reduction of -log$EC_{50}$ value, from 7.80 ± 0.11 for the control to 7.19 ± 0.02 for SJ-50 and to 6.94 ± 0.17 for SJ-100, corresponding to a dose-ratio of 12.84 ± 4.20 and 19.51 ± 11.14, respectively (Table 1). The inhibitory effects of SJ-50 and SJ-100 were abolished in the presence of $TXA_2$/PG receptor antagonist, $10^{-5}$M ONO-3708 (Fig. 4C). There was no difference in the -log$EC_{50}$ values for SJ-50 and SJ-100 relative to the control (6.01 ± 0.04 with SJ-50 and 6.02 ± 0.06 with SJ-100 vs. 6.02 ± 0.04 for the control), and the dose-ratio was reduced 1.03 ± 0.08 and 1.09 ± 0.02, respectively (Table 1).

***4.5.*** *Effects of plant extracts on BK-induced contraction*

The effects of SJ and GB on the contraction and relaxation response to $10^{-6}$ M BK are shown in Fig. 5A and Fig. 5B. As shown in Fig. 5A, SJ significantly and dose-dependently inhibited the contraction induced by $10^{-6}$ M BK, but did not alter the relaxation response (Fig. 5A). GB had no effect on BK-induced contraction or relaxation (Fig. 5B). SJ-100 significantly suppressed the magnitude of contraction induced by $10^{-6}$M BK (Fig. 5A). In the presence of $10^{-4}$ M L-NA, BK induced only contraction, and the effects of SJ and GB on this contraction are shown in Fig. 5C and 5D. SJ had a dose-dependent inhibitory effect (Fig. 5C), whereas GB had no effect (Fig. 5D). SJ-100 induced a significant and parallel shift of the presence curve to the right without suppression of

maximum contraction (Fig. 5C). When plotted as a percentage of the original maximum response, SJ-100 displaced the BK-induced CRC to the right with a significant reduction of the -log$EC_{50}$ value, from 7.83 ± 0.14 for the control to 5.93 ± 0.18, corresponding to a dose-ratio of 52.71 ± 20.29 (Table 1). The inhibitory effect of SJ-100 was abolished in the presence of $10^{-5}$M ONO-3708 (Fig. 5E), and addition of a $B_2$ antagonist (HOE140, $10^{-7}$ M) to SJ-100 did not alter the effect of SJ (Fig. 5E). There was no difference between the -log$EC_{50}$ value for SJ-100 and that of the control (5.27 ± 0.17 vs. 5.50 ± 0.25), and the dose-ratio was reduced to 1.98 ± 0.54 (Table 1).

***4.6.*** *Effects of plant extracts on $PGF_{2\alpha}$-induced contraction and BK-enhanced $PGF_{2\alpha}$ production*

The effects of SJ on $PGF_{2\alpha}$-induced contraction are shown in Fig. 6A and Fig. 6B. As seen in Fig. 6A, SJ significantly and dose-dependently inhibited the contraction induced by $PGF_{2\alpha}$. SJ-100 significantly shifted the $PGF_{2\alpha}$ response curve to the right without suppression of the maximum contraction (Fig. 6A). When plotted as a percentage of the original maximum response, SJ-100 displaced the $PGF_{2\alpha}$-induced CRC to the right was evident with a reduction of -log$EC_{50}$ value, from 7.09 ± 0.12 for the control to 6.71 ± 0.23, corresponding to a dose-ratio of 2.40 ± 0.45 (Table 1). The inhibitory effect of SJ-100 was abolished in the presence of $10^{-5}$ M ONO-3708 (Fig. 6B). There was no difference between the -log$EC_{50}$ for SJ-100 and that of the control (6.61 ± 0.17 vs. 6.61 ± 0.07), and the dose-ratio was reduced to 1.06 ± 0.41 (Table 1). The effect of SJ on BK-enhanced

$PGF_{2\alpha}$production by cultured PBAECs is shown in Fig. 6C. SJ-100 pre-treatment significantly suppressed the enhancement of BK-enhanced $PGF_{2\alpha}$ production ($p<0.01$).

## 5. DISCUSSION

Our present results obtained using PBA indicate that SJ has an inhibitory effect on contraction induced by histamine, 5-HT, U46619 and BK, and that GB has inhibits on histamine-induced contraction. Neither SJ nor GB had any effect on contraction induced by KCl and extracellular $Ca^{2+}$. Because $K^+$-induced contraction is the result of increased $Ca^{2+}$ influx through $Ca^{2+}$ channel and such extracellular $Ca^{2+}$-induced contraction response is usually studied when examining the inhibition targeting of extracellular $Ca^{2+}$ channels [62], our results ruled out the possibility that the effects of SJ and GB involve inhibition of extracellular $Ca^{2+}$ influx. At appropriate concentrations, GB shifted the CRC for histamine, and SJ shifted the CRCs for histamine, 5-HT, U46619 and BK to the right without altering the maximum contraction, suggesting that antagonism on mediating receptors might be involved in the responses [5]. In addition, significant reduction in $-\log EC_{50}$ values and the increased dose-ratios added further evidence for parallel displacement [95]. Because we had ruled out the participation of $Ca^{2+}$ influx inhibition in the response to SJ and GB extracts, it was possible that the inhibitory effects were due to targeting of receptors mediating contraction in response to the agonists examined.Previous studies conducted by our group using PBA have clarified that receptor $H_1$ mediates histamine contraction, receptors 5-$HT_1$ and 5-$HT_2$ mediate 5-HT contraction, and receptor $TXA_2$/PG mediates

U46619 and BK contraction, and that antagonists, including $10^{-5}$ M diphenhydramine, $10^{-6}$ M ketanserin and $10^{-5}$ M ONO-3708 are sufficient to block these receptors, respectively [85, 88, 90]. Therefore we verified the involvement of $H_1$, 5-$HT_2$ and$TXA_2$/PG receptors in the inhibition effects of SJ and GB by re-examining them with the blockade of these receptors. Pre-treatment with $10^{-5}$ M diphenhydramine abolished the competitive inhibitory effect of GB and SJ on histamine-induced contraction, demonstrating that antagonism at the $H_1$ receptor had mediated the inhibition. Pre-treatment with $10^{-6}$M ketanserin abolished the competitive inhibitory effect of SJ on 5-HT-induced contraction, demonstrating and identifying that this effect was attributable to antagonism of the 5-$HT_2$, but not the 5-$HT_1$ receptor. Because U46619 acts as a $TXA_2$/PG receptor agonist, the abrogation of SJ competitive inhibition by pretreatment with the antagonist ONO-3708 ($10^{-5}$ M) confirmed the antagonistic effect of SJ on the $TXA_2$/PG receptor. When testing the effects of the plant extracts on the biphasic response to BK, we observed that SJ significantly suppressed the contraction, but had no effect on the relaxation induced by BK. A previous study by our group using PBA had identified that the only one kind of BK receptor ($B_2$) was located on PBA and responsible for both contraction and relaxation induced by BK, and that antagonism on this $B_2$ receptor simultaneously suppressed both of these responses [88]. Therefore, the selective effect of SJ on only BK-induced contraction in this biphasic response ruled out the possibility that SJ inhibition had targeted on the $B_2$ receptor. In addition, because $PGH_2$ released from endothelial cells upon BK stimulation was identified as the main factor contributing to BK-induced contraction [88], it was possible that inhibition of $PGH_2$-mediated pathway in response to BK was involved in SJ effect. As

ONO-3708 ($10^{-5}$ M) had been demonstrated to abolish the participation of $PGH_2$-mediated contraction in the response to BK [88], we applied this antagonist to verify our hypothesis. We found that ONO-3708 ($10^{-5}$ M) abolished the effect of SJ on BK, thus proving that the inhibitory effect of SJ on BK-induced contraction had targeted on $PGH_2$-mediated pathway. Furthermore, when ONO-3708 ($10^{-5}$ M) was applied, the presence of a $B_2$ antagonist (HOE140, $10^{-7}$ M) did not increase the effect of SJ-100 further, confirming that inhibition of the $PGH_2$ pathway was the main mechanism responsible for the inhibitory effect of SJ on BK-induced contraction. Our group has recently clarified a role for the $PGH_2$-mediated pathway in BK-induced contraction of PBA, in which the short-lived metabolite $PGH_2$ released by BK stimulation was later converted to active vasoconstrictive $PGF_{2\alpha}$,thusincreasing$PGF_{2\alpha}$ production and evoking contraction [58]. Therefore, we hypothesized that the inhibitory effect of SJ on the $PGH_2$-mediated contraction pathway involved in BK response might be mediated through inhibition on BK-enhanced $PGF_{2\alpha}$ production and-or inhibition of $PGF_{2\alpha}$-induced contraction. The effect of SJ on BK-enhanced $PGF_{2\alpha}$ production was evaluated using cultured PBAECs. Similarly to our previous study [58], the results showed that PBAECs spontaneously produced $PGF_{2\alpha}$, and that BK ($10^{-7}$ M) significantly enhanced this $PGF_{2\alpha}$ production; however, this enhanced $PGF_{2\alpha}$ production was significantly suppressed by SJ. SJ also significantly inhibited $PGF_{2\alpha}$-induced contraction. Because no study has established mechanisms and identified receptors mediating $PGF_{2\alpha}$ contraction on PBA, we were unable to examine in the full scale the effect of SJ on all receptors mediating $PGF_{2\alpha}$ contraction. However, previous researches using blood vessels, including cerebral arteries, have suggested that the $TXA_2$/ PG

receptorparticipates to induce $PGF_{2\alpha}$ contraction, and that the antagonist ONO-3708 is able to block this receptor participation [65, 128]. In addition, when testing SJ against U46619-induced contraction, we had demonstrated that SJ antagonized the $TXA_2$/PG receptor. Therefore we hypothesized that antagonism of this receptor might also mediate the SJ-induced inhibition of $PGF_{2\alpha}$-induced contraction and applied ONO-3708 ($10^{-5}$ M) to re-examine this effect. As expected, ONO-3708 ($10^{-5}$ M) abolished the inhibitory effect of SJ, thus confirming that antagonism of the $TXA_2$/PG receptor had mediated the effect of SJ on $PGF_{2\alpha}$ contraction. Considering these results overall, we concluded that a synergistic inhibitory effect of SJ on BK-enhanced $PGF_{2\alpha}$ production and the $TXA_2$/PG receptor was responsible for the effect of SJ on BK-induced contraction. However, because there has been no information on the precise mechanisms of $PGF_{2\alpha}$ synthesis and the nature of the enzymes responsible for the transformation of $PGH_2$ to $PGF_{2\alpha}$ in PBA, the factors or pathways involved in the inhibitory effect of SJ on this conversion remain to be determined. In addition, as BK is a pro-inflammatory peptide that mediates a variety of physiological and pathological responses [43, 72, 73], it is also possible that inhibitory effects of SJ on BK-induced responses might play roles in many other pharmacological functions of SJ, and the further researches aretherefore needed to elucidate these potentials.

Present study represents the first attempt to test the receptor antagonism of SJ and GB on isolated cerebral artery. Our study demonstrated that GB had an inhibitory effect on PBA contraction induced by histamine, and that SJ inhibited the contraction induced by histamine, 5-HT, U46619 and BK. Because it is well established that those agonists participate to induce cerebral spasm in pathological conditions [81, 116], interaction of SJ

and GB on these contractions provide evidences to clarify their potential to reduce spasm, and thus explaining their therapeutic use in diseases related to cerebrovascular spasm. It has been reported previously that the effect of GB on cerebral spasm is mediated via suppression on ET-1 over-expression [123], release of nitric oxide [21] and the effects of antioxidants [69]. The inhibition of histamine-induced contraction by GB demonstrated in this study has further clarified the mechanism responsible for the anti-vasospasm effect of GB. The inhibitory effects of SJ and GB against vasospasm were partly attributable to their antagonistic effects on receptors mediating vasoconstriction, including the $H_1$, 5-$HT_2$ and $TXA_2$/PG receptors. Because many mediators of cerebral vasospasm have been identified and various antagonists of these mediators have been shown to ameliorate cerebral spasm [130, 131], the antagonistic effects of SJ and GB on such receptors identified in the present study might also have potential therapeutic application.Here we found that SJ was more potent in this respect than GB, the former demonstrating antagonism of $H_1$, 5-$HT_2$ and $TXA_2$/PG receptors, whereas the latter acted only on the $H_1$ receptor. Many studies have demonstrated that potential vasoconstrictors, released from activated platelets and damaged erythrocytes, participate in cerebral vasospasm [66]. $TXA_2$ and 5-HT, both released from platelets in the response to hemorrhage, are powerful vasoconstrictors and have been widely reported to play important roles in SAH-induced cerebral vasospasm [115, 125]. Hemorrhage pathogenesis is demonstrated to not only significantly increase the levels of $TXA_2$ and 5-HT [97, 112] but also remarkably potentiate their contraction potency [59, 98]. It has been suggested that antagonists of $TXA_2$ and 5-$HT_2$ receptors might have potential therapeutic application for cerebral vasospasm [130, 131]. Since $TXA_2$ and 5-HT are two

major putative spasmogens whose levels and potency increase markedly by the response to hemorrhage, SJ antagonism of their mediating receptors ($TXA_2$/PG and 5-$HT_2$ receptors) would certainly play important roles in the treatment of vasospasm associated with hemorrhage, such as SAH. Therefore the present results provide a partial pharmacological explanation for the reputed therapeutic efficacy of SJ for cerebral hemorrhage described in Vietnamese traditional medicine [28, 74]. Although several recent studies have identified that SJ and GB contain a number of common components, only GB has been widely researched and applied for treatment of cerebral disorders [71]. GB is one of the best-selling herbal supplements in Europe and the United States, and there has been hundreds of published studies investigating GB effect on functions of the brain [17]. However, in contrast to GB, SJ usage for cerebral disorders has been based mainly on folk experiences [28, 74], and research into its effect has been limited to only cerebral infarction [20, 71]. Because, in accordance with Vietnamese traditional therapy, our study has revealed that SJ might be a promising candidate for treatment of cerebral vasospasm associated with hemorrhage, we suggest that further research to exploit its potential would be justified.

Similar to SJ in the present study, concurrent antagonism on two or more receptors has been demonstrated to mediate pharmacological functions of medicinal plants [64, 67]. Mechanism by which a plant exerts multi-receptor antagonism has not yet been established, but research suggested that the presence of several, and not one, active components is responsible for this mode of action[67]. SJ and GB have been reported to contain components such as rutin, quecertin, genistein and kaempferol[20], which are able to cross the brain-blood barrier [54, 108, 129, 141]and can suppress contraction due to

vasoconstrictors [16, 25]. Our study has not yet isolated active components of SJ and GB to investigate their antagonism so far, but it will be underway by future research.In addition, because blood-brain barriers is an important mechanism for protecting the brain from fluctuations of compositions in plasma and from circulating agents [9, 10], the *in vivo* experiments to access how the active components of plants cross through the blood-brain barriers are especially required in researching about their uses for brain disorders. Although the mechanisms of blood-brain barrier dysfunction after neurological disease remain to be further understood, the change of blood-brain barrier permeability after stroke of brain was accepted widely [27], and previous studies has indicated that medicinal plants, such as GB, could pass through blood-brain barrier in pathological conditions, for example,after ischemia-reperfusion injury of brain [35], and therefore support the use of plants for the cerebral disorders[52]. In addition, the SJ capable of targeting multiple receptors at the same time might fulfill a multiple-target strategy of future drugs, and therefore we believe that further study to identify the components, as well as their crossing through blood-brain barriers in pathological conditions, would be worth to exploit this promissory herb applications in cerebral hemorrhage.

## 6. CONCLUSIONS

Using PBA, our present study has shown that SJ and GB exert inhibitory effects on the vasoconstriction induced by various agents. The effects of SJ and GB are attributed to antagonism of the $H_1$, 5-$HT_2$ and $TXA_2$/PG receptors, and provide an explanation for their

therapeutic use for cerebral disorders. SJ exerts more potent antagonism than GB. In addition, because the antagonism of SJ on 5-$HT_2$ and $TXA_2$/PG receptors plays a more important role in the treatment when cerebral vasospasm is associated with hemorrhage, it partly explains why SJ has been used traditionally in Vietnamese ethnic medicine for treatment of cerebral hemorrhage.

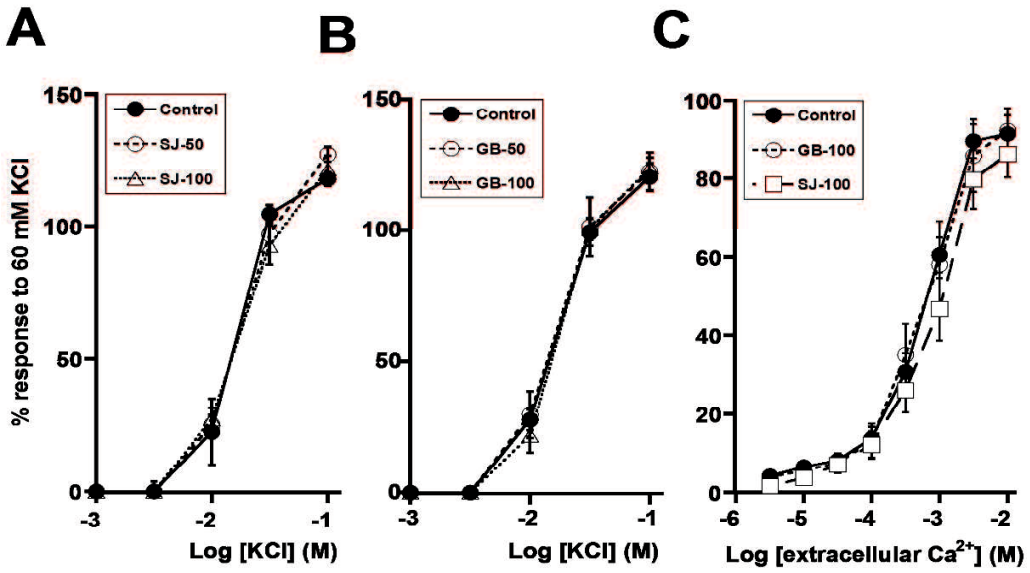


**Fig 1. Effects of *Sophora japonica* L. and *Gingko biloba* on KCl-induced contraction [A and B]and on extracellular $Ca^{2+}$-induced contraction [C]of PBA rings.** SJ-50, SJ-100 and GB-50, GB-100 in [A], [B] and [C] figures respectively represent the application of *Sophora japonica* L. (SJ) and *Gingko biloba* (GB) extracts at concentrations of 50 µl and 100 µl per 5 ml organ bath fluid. Contraction induced by 60 mMKCl (7.65 ± 0.19 mN) before addition of plant extract was taken as 100%. Each point represents the mean ± SEM for 6 different pigs.

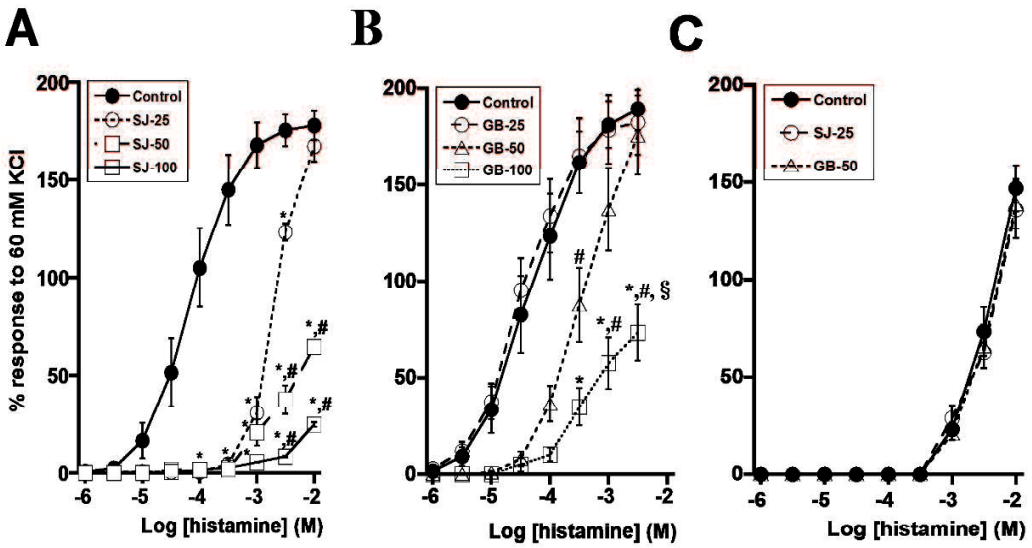


**Fig 2. Effects of *Sophora japonica* L. and *Gingko biloba* on histamine-induced contraction of PBA rings in the absence [A and B] and the presence [C] of $10^{-5}$ M diphenhydramine.** SJ-25, SJ-50, SJ-100 and GB-25, GB-50, GB-100 in [A], [B] and [C] figures respectively represent the application of *Sophora japonica* L. (SJ) and *Gingko biloba* (GB) extracts at concentrations of 25 µl, 50 µl and 100 µl per 5 ml organ bath fluid. The responses at each concentration in the absence and presence of different extract concentrations were compared by one-way ANOVA followed by the Bonferroni test (in [A]: $^{*}p<0.05$ vs. Control; $^{\#}p<0.05$ vs. SJ-25, in [B]: $^{*}p<0.05$ vs. Control, $^{\#}p<0.05$ vs. GB-25, $^{\S}p<0.05$ vs. GB-50). Contraction induced by 60 mMKCl (7.72 ± 0.20 mN) before addition of plant extract was taken as 100%. Each point represents the mean ± SEM for 6 different pigs.

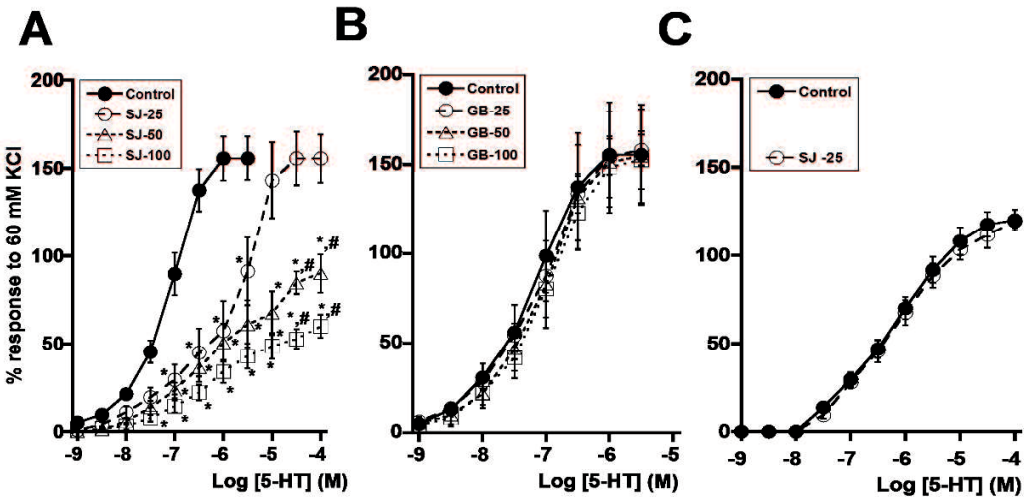


**Fig 3. Effects of *Sophora japonica* L. and *Gingko biloba* on 5-hydroxytryptamine-induced contraction of PBA rings in the absence [A and B]and the presence [C]of $10^{-6}$ M ketanserin.** SJ-25, SJ-50, SJ-100 and GB-25, GB-50, GB-100 in [A], [B] and [C] figures respectively represent the application of *Sophora japonica* L. (SJ) and *Gingko biloba* (GB) extracts at concentrations of 25 µl, 50 µl and 100 µl per 5 ml organ bath fluid. The responses at each concentration in the absence and presence of different extract concentrations were compared by one-way ANOVA followed by the Bonferroni test or Student's *t*-test (in [A]: $^{*}p<0.05$ vs. Control; $^{\#}p<0.05$ vs. SJ-25). Contraction induced by 60 mMKCl (7.60 ± 0.11) before addition of plant extract was taken as100%. Each point represents the mean ± SEM for 6 different pigs.

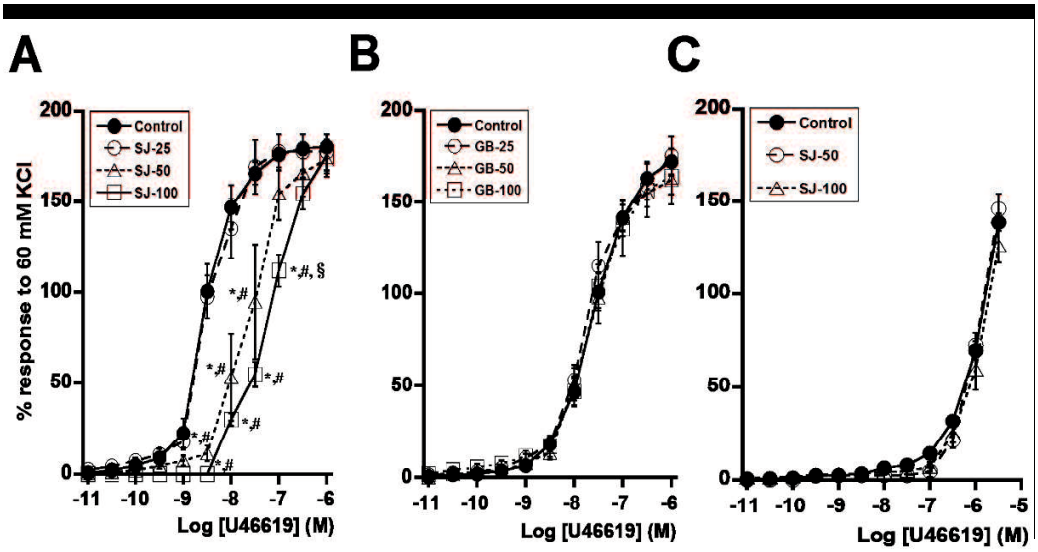


**Fig 4. Effects of *Sophora japonica* L. and *Gingko biloba* on U46619-induced contraction of PBA in the absence [A and B]and the presence [C] of $10^{-5}$ M ONO-3708. SJ-25, SJ-50, SJ-100 and GB-25, GB-50, GB-100 in [A], [B] and [C] figures respectively represent the application of *Sophora japonica* L. (SJ) and *Gingko biloba* (GB) extracts at concentrations of 25 µl, 50 µl and 100 µl per 5 ml organ bath fluid.** The responses at each concentration in the absence and presence of different extract concentrations were compared by one-way ANOVA followed by the Bonferroni test (in [A]: $^{*}p<0.05$ vs. Control; $^{\#}p<0.05$ vs. SJ-25; $^{\S}p<0.05$ vs. SJ-50). Contraction induced by 60 mMKCl (7.63 ± 0.21 mN) before addition of plant extract was taken as 100%. Each point represents the mean ± SEM for 6 different pigs.

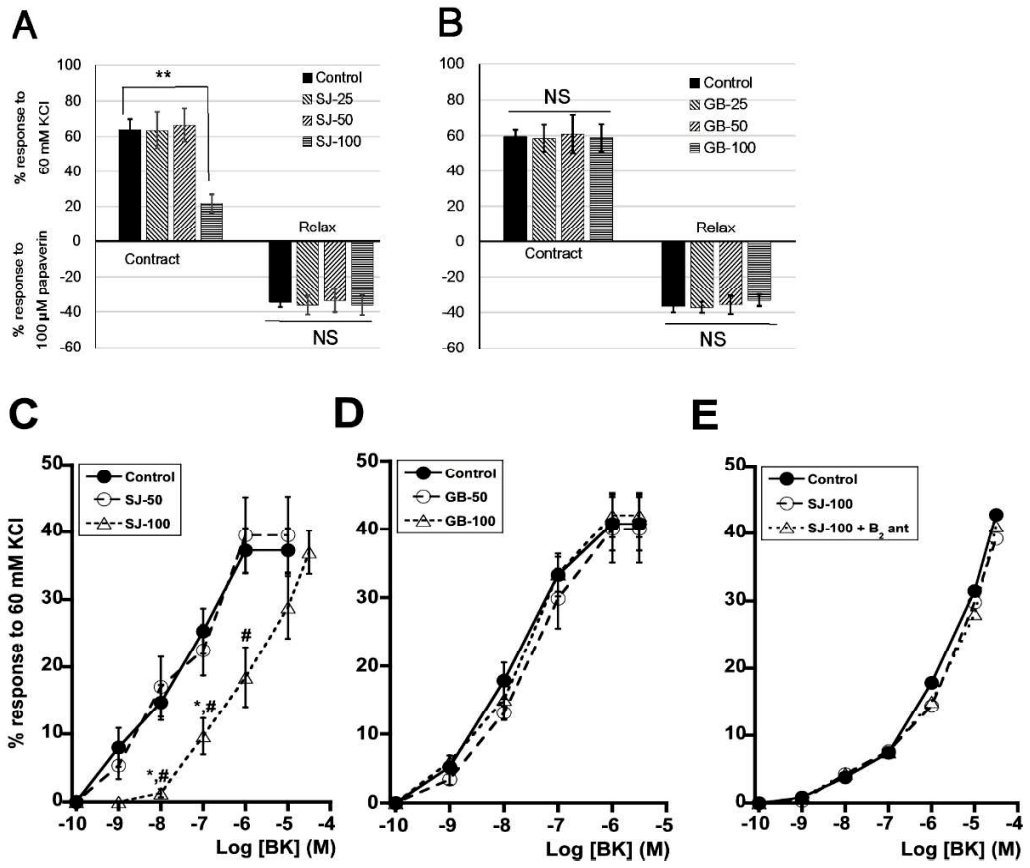


**Fig 5. Effects of *Sophora japonica* L. [A]and *Gingko biloba* [B]on the bradykinin-induced biphasic response. SJ-25, SJ-50, SJ-100 and GB-25, GB-50, GB-100 in [A], [B], [C], [D] and [E] figures respectively represent the application of *Sophora japonica* L. (SJ) and *Gingko biloba* (GB) extracts at concentrations of 25 µl, 50 µl and 100 µl per 5 ml organ bath fluid.** Theabsolute values of KCl-induced contraction andpapaverine-induced relaxation were 7.77 ± 0.19 mN and 5.15 ± 0.11 mN, respectively, and were taken as the 100% contraction and relaxation responses. Effect of *Sophora japonica* L. and *Gingko biloba* on BK-induced contraction in the presence of $10^{-4}$ M L-NA in the absence [C and D] and in the presence [E] of $10^{-5}$ M ONO-3708. The responses at each concentration in the absence and presence of different extract concentrations were compared by one-way ANOVA followed by the Bonferroni test (in [A, B]: $^{**}p<0.01$ vs. Control, NS: not significant vs. Control, in [C]: $^{*}p<0.05$ vs. Control; $^{\#}p<0.05$ vs. SJ-50). Contraction induced by 60 mMKCl (7.98 ± 0.12 mN) before addition of plant extract was taken as 100%. Each point represents the mean ± SEM for 6 different pigs.

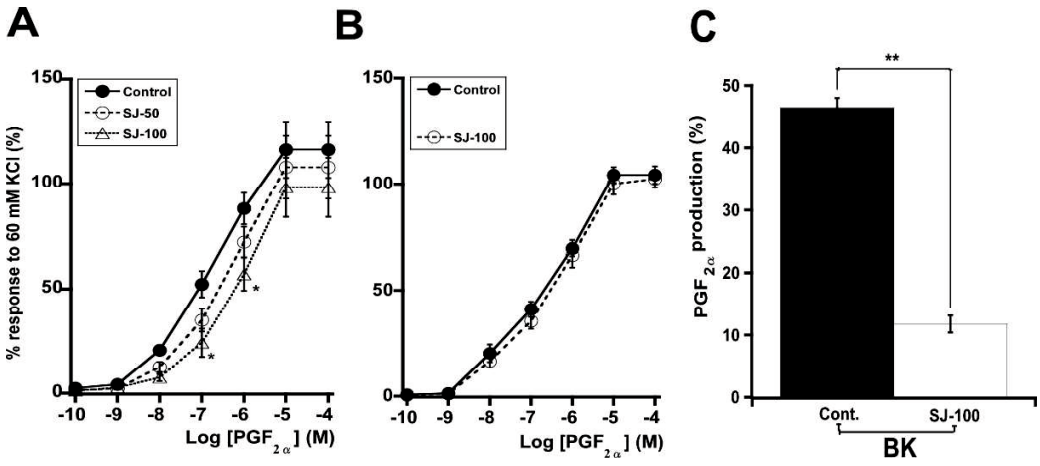


**Fig 6. Effects of *Sophora japonica* L. on $PGF_{2\alpha}$-induced contraction of PBA rings in the absence[A] and the presence [B] of $10^{-5}$ M ONO-3708. SJ-50 and SJ-100 in [A], [B] and [C] figures respectively represent the application of *Sophora japonica* L. (SJ) extract at concentrations of 50 µl and 100 µl per 5 ml organ bath fluid.** Contraction induced by 60 mMKCl (7.81 ± 0.22 mN) before addition of plant extract was taken as 100%. Each point represents the mean ± SEM for 6 different pigs. Effect of *Sophora japonica* L. on BK ($10^{-7}$ M)-enhanced $PGF_{2\alpha}$ production from cultured PBA endothelial cells after 1 h of treatment[C]. Spontaneous production without BK stimulation (8.22 ± 0.55 pg/$10^5$ cells) was taken as 100%. Each point represents the mean ± SEM for 4 different pigs and three independent experiments in duplicate. $^{**}p<0.01$ vs. Control by Student's *t*-test.

| | | Without receptor antagonist | | With receptor antagonist | |
|---|---|---|---|---|---|
| | | -LgEC$_{50}$ | Dose-ratio | -LgEC$_{50}$ | Dose-ratio |
| **Histamine** | | Without diphenhydramine | | With $10^{-5}$ M diphenhydramine | |
| | Control | 4.42 ± 0.09 | | 2.49 ± 0.04 | |
| | SJ-25 | 2.74 ± 0.05*** | 35.21 ± 12.86 | 2.49 ± 0.12 | 1.07 ± 0.12 |
| | GB-50 | 3.36 ± 0.11*** | 8.59 ± 2.51 | 2.48 ± 0.03 | 1.01 ± 0.03 |
| **5-hydroxytryptamine** | | Without ketanserin | | With $10^{-6}$ M ketanserin | |
| | Control | 6.98 ± 0.15 | | 6.14 ± 0.18 | |
| | SJ-25 | 5.48 ± 0.21*** | 85.98 ± 3.54 | 6.07 ± 0.22 | 1.21 ± 0.15 |
| **U46619** | | Without ONO-3708 | | With $10^{-5}$ M ONO-3708 | |
| | Control | 7.80 ± 0.11 | | 6.02 ± 0.04 | |
| | SJ-50 | 7.19 ± 0.02* | 12.84 ± 4.20 | 6.01 ± 0.04 | 1.03 ± 0.08 |
| | SJ-100 | 6.94 ± 0.17* | 19.51 ± 11.14 | 6.02 ± 0.06 | 1.09 ± 0.02 |
| **Bradykinin** | | Without ONO-3708 | | With $10^{-5}$ M ONO-3708 | |
| | Control | 7.83 ± 0.14 | | 5.50 ± 0.25 | |
| | SJ-100 | 5.93 ± 0.18* | 52.71 ± 20.29 | 5.27 ± 0.17 | 1.98 ± 0.54 |
| **PGF$_{2\alpha}$** | | Without ONO-3708 | | With $10^{-5}$ M ONO-3708 | |
| | Control | 7.09 ± 0.12 | | 6.61 ± 0.17 | |
| | SJ-100 | 6.71 ± 0.23 | 2.40 ± 0.45 | 6.61 ± 0.07 | 1.06 ± 0.41 |

**Table 1. Effect of *Sophora japonica* L. and *Gingko biloba* on -logEC$_{50}$ and dose-ratio values of the contraction induced by histamine, 5-hydroxytryptamine, U46619, bradykinin and prostaglandin F$_{2\alpha}$ on porcine basilar artery, in the absence and the presence of receptor antagonists.** SJ-25, SJ-50, SJ-100 respectively represent the application of *Sophora japonica* L. (SJ) extract at concentrations of 25 µl, 50 µl and 100 µl per 5 ml organ bath fluid, and GB-50 represent the application of *Gingko biloba* (GB) extract at concentrations of 50 µl per 5 ml organ bath fluid. -logEC$_{50}$ was computed from the response expressed as a percentage of the maximum response in each concentration response curve. The -logEC$_{50}$ value obtained from the concentration response curve constructed in the presence of extract was compared with that in the absence of extract by Student's *t* test ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. control). Each value represents the mean ± SEM for 6 different pigs.

# General discussion

The chapter 1, study 1, characterized the pharmacological responsiveness of AVL on isolated PBA, and showed that AVL has biphasic effect on arteries, which partly explains both its indication and contraindication for treatment of hypertension. This biphasic effect of AVL is mediated, at least in part, by both stimulation and inhibition of $Ca^{2+}$ channels. It is likely that under resting tension, their inhibition of $Ca^{2+}$ channels was overwhelmed by stimulation, but when the $Ca^{2+}$ channels had already been stimulated, antagonistic effects were expressed. Our study has not isolated active components responsible for this mode of biphasic actions, however, components exerting a biphasic effect on $Ca^{2+}$ channels, such as quercetin, have been identified in AVL by previous researches. The presence of those components in AVL might contribute to its biphasic effect, but next studies clarify their roles are necessary to give an insight in the AVL biphasic responses. In this study, for the first time, our present study has determined that 5-HT as a component of AVL. Our present study demonstrated that 5-HT in AVL contributes to the plant-induced contraction effect on isolated artery, but has not yet determined the role of 5-HT in the plant-induced *in vivo* vascular effect, which is required to be clarified in future studies. In addition, besides effect on the vascular system, AVL has also been traditionally applied fordisorders on the central nervous system, and is traditionally believed to induce analgesic, sedative, anti-depressant and antiepileptic effects. 5-HT has been known to have many important anti-depressant pathways, therefore it is possible that 5-HT contributes to the anti-depressant effect of AVL, because the neurological activity of

many other medicinal plants had been attributed to their content of 5-HT and/or 5-HT precursor components. We believe that further investigation would be justified to access the role of 5-HT in AVL antidepressant use.

The chapter 2, study 2, characterized the pharmacological responsiveness of GB and SJ on cerebral vasoconstriction in response to histamine, 5-hydroxytryptamine, U46619 and bradykininof isolated PBA, and showed that SJ and GB exert inhibitory effects on the vasoconstriction induced by those agents. The inhibitory effects of SJ and GB against those spasmogens were partly attributable to their antagonistic effects on the receptors that mediate vasoconstriction, such as $H_1$, 5-$HT_2$ and $TXA_2$/PG receptors. As many mediators of cerebral vasospasm have been identified and various antagonists of these mediators have been shown to ameliorate cerebral spasm, the antagonistic effects of SJ and GB on such receptors identified in the present study partly provide an explanation for their therapeutic use in cerebral disorders related to vasospasm of brain artery. SJ exerts more potent antagonism than GB, because the former demonstrated antagonism of $H_1$, 5-$HT_2$ and $TXA_2$/PG receptors, whereas the latter acted only on the $H_1$ receptor.Since $TXA_2$ and 5-HT are two major putative spasmogens whose levels and potency increase markedly by the response to hemorrhage, SJ antagonism of their mediating receptors ($TXA_2$/PG and 5-$HT_2$ receptors) would certainly play important roles in the treatment of vasospasm associated with hemorrhage, such as SAH. This result explains, at least in part, the reputed therapy of SJ in cerebral hemorrhage, which has been described in Vietnamese traditional medicine. In addition, SJ capable of targeting multiple receptors at the same time might make this plant material become a candidate for future drugs, which are expected to possess themultiple-

target strategyin disease treatments.Future study to identify active components of SJ would provide aninsight into its promissory potential. Because plant extracts are known to contain many compounds, the establishment of active constituents in plant materialsfrom SJ and GB are necessary to further access their effects, which have been preliminarily detected in this study.

# General Conclusion

In conclusions, the present thesis verified the effects of AVL, SJ and GB on PBA, and from that give pharmacological basis to explain the contraindications of AVL in hypertension, and the therapeutic uses of SJ and GB in cerebral hemorrhage and other diseases associated with cerebral vasospasm in traditional medicine. The results demonstrated pharmacological characteristics of those plants and partly explained their treatment effects, but further researches are necessary to support their potentials and propose the safe applications. In addition, our data have not address the *in vivo* results, so it remained to bedeterminedwith future researches. We expect that our next studies, which investigate the effects of those plants *in vivo* and in different pathological states wouldprovide more evidences to exploit their therapeutic uses.

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