Raghava Naidu Sriramaneni et al /J. Pharm. Sci. & Res. Vol.4(8), 2012, 1924-1928

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Chronic Effects of *Andrographis paniculata* Chloroform Extract in Spontaneously Hypertensive Rats

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Abstract

This study was undertaken to investigate the effects of chronic administration of chloroform extract of *Andrographis paniculata,* known to possess some cardioprotective action, on endothelial dysfunction using isolated spontaneously hypertensive (SH) rat thoracic aorta. Verapamil hydrochloride was used as a positive control to compare its effect on endothelial dysfunction to that of *Andrographis paniculata* chloroform extract (APCE). HPLC study of APCE was performed and compared with that of the standards, andrographolide (ANG), and 14-deoxyandrographolide (DA). Adult male SH rats were treated with 25, 50 and 100 mg/kg APCE and 12.5 mg/kg Verapamil (p.o.) once a day for four weeks. There was a significant ($P<0.05$) reduction in tail-cuff systolic blood pressure (SBP) measurements in conscious animals treated with 50 and 100 mg/kg APCE as well as in those treated with Verapamil. Similarly, these groups showed endothelium-dependent relaxation to acetylcholine (ACh). Animals treated with APCE 100 mg/kg and Verapamil hydrochloride 12.5 mg/kg showed endothelium-independent relaxation to sodium nitroprusside (SNP). The findings collectively suggest that chronic treatment with APCE preserves vascular endothelial functions in SH rats.

Key words; Acetylcholine, aorta, diterpenoid lactones, endothelium dysfunction, sodium nitro prusside, spontaneously hypertensive rats.

INTRODUCTION

Andrographis paniculata (F. *Acanthaceae*) is a medicinal plant in Malaysia which possesses potent effects in treating diabetes and hypertension. (Ahmad and Asmawi, 1993). Studies have shown that *Andrographis paniculata* (AP) has therapeutic actions on the cardiovascular system (Chang and But, 1987). Water extract obtained from AP produces a significant reduction in systolic blood pressure in both normotensive and SH rats (Zhang, 1996; Tan, 1997). Diterpenoid lactones isolated from AP, namely deoxyandrographolide (DA) and 14-deoxy-11, 12-didehydroandrographolide (DDA), have been shown to cause a significant fall in mean arterial blood pressure (MABP) and heart rate. Moreover, these lactones are scientifically proven to non-competitively and dose-dependently antagonize isoproterenol-induced positive chronotropic actions in rat atria (Zhang *et al*., 1998). In isolated rat thoracic aorta, DA has been observed to alter calcium homeostasis, reduce the contractile response of $CaCl_2$ in the presence of high K^+ and ultimately contribute to vasorelaxation (Zhang and Tan, 1998). Besides AP able to selectively block voltage operated calcium channels and hence inhibiting the calcium influx (Burgos *et al*., 2000).

Vascular endothelial dysfunction is thought to contribute to the development of various cardiovascular disorders such as impairment in coronary circulation and hypertension. (Rubanyi *et al*., 1993; Luscher *et al*., 1994). Various mechanisms may be involved in the development of endothelial dysfunction and eventually cardiovascular diseases. Antihypertensive drugs, such as angiotensin-converting enzyme inhibitors, calcium-channel blockers, (Rodrigo *et al*., 1997; Novosel *et al*., 1994), and antioxidants are well known to improve endothelial dysfunction (Ulker *et al*., 2003; Levine *et al*., 1996).

Besides its pharmacological effects on the cardiovascular system, AP possesses other pharmacological activities, such as antipyretic, anti-inflammatory (Deng *et.al*., 1982), hepatoprotective (Shukla et al., 1992 ; Kapil *et al*., 1993), immunostimulantory (Puri *et al*., 1993), and antioxidant actions (Akowuah *et al*., 2006). Based this background, the main aim of the present study was to investigate the effects of chronic administration of different doses of APCE on systolic blood pressure and vascular function on isolated rat aortas of SH rats.

MATERIALS AND METHODS

Plant material

Fresh aerial parts of *Andrographis paniculata* were collected from the cultivated nurseries of Malaysian Agriculture Development Institute, Kelantan, Malaysia and authenticated

by the School of Biological Sciences, Universiti Sains Malaysia.

Preparation of the plant's crude extracts

The dried aerial parts of *Andrographis paniculata* were powdered and successively extracted with petroleum ether (60 to 80ºC) for a period of 48 h, chloroform for a period of 72 h and methanol for 72 h using Soxhlet apparatus. The powdered plant material was allowed to dry up before commencing a new extraction process using different solvent. All extracts were evaporated to dryness using Buchi Rotavapor (Switzerland) and subsequently freeze-dried. The chloroform extract was found to possess the most potent vasorelaxant effects against nor-epinephrine induced contractions in isolated rat thoracic aorta (Naidu *et al*., 2007).

Experimental Animals

Male SH rats aged about 10-12 weeks and weighing between 230-310 g were used in this study. The animals were bred and housed in the animal house, School of Pharmaceutical Sciences, University Sains Malaysia (USM). Animals were allowed a free access to food (normal laboratory chow, Gold Coin) and tap water *ad libitum*. This study was carried out in accordance with the National and International guidelines for experimental animal handling and was approved by University Science Malaysia Animal Ethics Committee for the use of experimental animals. The animals were divided into following groups: Group 1, control untreated group which received normal saline. Group 2 received Verapamil 12.5 mg/kg b.w, and groups 3, 4 and 5 received APCE at doses of 25, 50 and 100 mg/kg b.w. respectively. Group 6 received the vehicle only (0.1% Tween-80). All the preparations were administered orally and once daily for 4 weeks.

Drugs, chemicals and solutions

Petroleum ether (60-80 °C), chloroform and methanol were purchased from R & M Chem., UK. Verapamil hydrochloride, phenylepherine hydrochloride, acetylcholine hydrochloride (ACh), Tween- 80 were obtained from Sigma Chemical Co, St Louis, MO. Sodium nitroprusside were purchased from BDH, laboratory supplies, England. APCE was first suspended in (10% v/v) Tween 80 and the resultant solution was completed to the required volume with distilled water.

Chronic effects of APCE on noninvasive systolic blood pressure measurement

In this part of study, the effect of a four weeks chronic administration of daily oral doses of 25, 50 and 100 mg/kg body weight, APCE on blood pressure was measured. APCE was suspended in 10% (v/v) Tween 80. The stock solution was prepared once every three days. Extract suspensions were stored at 4ºC and were allowed to reach room temperature before administration.

Systolic blood pressure (SBP) measurement of SH rats was carried out using tail-cuff method plethysmography (NIBP machine, IITC Inc., CA, USA) in conscious pre-warmed (27-29°C for 30 min) restrained rats. During the final week of the treatment, the rats were allowed to acclimatize to the experimental conditions of noninvasive SBP measurements by allowing them to stand in rat restrainers for 30 min every day. SBP measurements were recorded 24 h after the last treatment dose. At least 8-10 recordings were taken for each rat and the mean of the lowest 4 values within less than 10 mmHg difference was taken as the mean SBP.

Chronic effects of APCE on rat aortic contraction

In this part of the study, the effects of chronic administration of APCE on vascular function in hypertension were examined using isolated rat thoracic aorta. Following the completion of the noninvasive systolic blood pressure measurement protocol, the animals were sacrificed by cervical dislocation and the thoracic aorta was carefully isolated, cleaned of fat and connective adipose tissue, and cut into 3 – 5 mm long rings. The rings were suspended horizontally in tissue chambers containing 10 mL of Kreb's physiological solution. Special care was taken to avoid damage to the endothelium. The tissue-bath solution was aerated with Carbogen gas at 37ºC (Ajay *et al*., 2005). Aortic rings were allowed to equilibrate at an optimal tension of 1 g for 30 min. During this period, Kreb's solution was replaced every 15 min and, if needed, the tension was readjusted to 1 g. During the stabilization period the aortic rings were exposed twice (each for 10 min) to isotonic potassium chloride solution (high K^+, 80 mM). After wash out of K^+, the aortic rings were contracted with phenylepherine (PE, 1 µM, 3 min), then responses to the actions of endothelium-dependent (ACh) and –independent (SNP) dilators were recorded. Cumulative dose relaxation curves were constructed with ACh (10 pM to 10 µM) and sodium nitroprusside (SNP) (1 pM to 0.1 µM). Responses were recorded isometrically via a force–displacement transducer (P23 ID Gould, Statham Instrument, UK) connected to Grass polygraph model 79D (Quincy, Mass., USA).

HPLC analysis

HPLC analysis was performed on a Shimadzu LC-10AT (Shimadzu Corporation, Kyoto, Japan) system equipped with a LC-6A solvent delivery pump equipped with a SPD-10A UV/VIS detector. Data were acquired and processed by Class-VP Chromato software. The analytical column used was Nucleosil C18 (250×4.6 mm i.d., 5 µm; Phenomenex) along with a Guard Column C18 (10×4.0 mm i.d; Phenomenex) used at ambient temperature for the elution of analyte. A mobile phase consisting of methanol: water (65:35 v/v) was prepared and filtered through 0.45 µm nylon-membrane filter (Millipore Corporation, MA, USA) under vacuum before use. Methanol (JT Baker Co, USA) used as the mobile phase was of HPLC-grade. The analysis was run at a flow rate of 1 mL/min. The detection was set at a wavelength of 223 nm.

Stock solutions of ANG and DA were prepared at a concentration of 1 mg/ml in mobile phase. The stock solutions were further diluted with the mobile phase to obtain the calibration standards of 10, 20, 30, 40, 50 and 100 µg/mL. A sample solution of 0.05% (w/v) APCE was prepared by dissolving 10 mg of the extract in 20 mL of mobile phase under ultrasonication for 25 min to obtain concentration of 500 µg/mL. This solution was further diluted with the mobile

phase to yield 250 µg/mL. The standards and the extract samples were injected (in triplicates) in a sample volume of 20 µ L. Before injection, the extract samples were filtered through, a 0.2-µm, polytetrafluoroethylene (PTFE) membrane (Aervent® Disposable Filters, Millipore Corporation). Active constituents of the APCE were identified by comparison of HPLC retention times of authenticated sample of DDA analyzed under identical conditions.

Data presentation and statistical analysis

Relaxation responses to cumulative concentrations of ACh and SNP were calculated as percentage inhibition of phenylephrine (PE) induced maximal contraction. Concentration-dependent contractile responses to PE were recorded as percentage of the maximum contraction obtained following tissue stimulation with high K^+. All the results were expressed as mean ± SEM of 6-8 experiments. The E_{max} and EC_{50} values were calculated by using a (GraphPad Prism version 5.01. USA). The values were plotted to obtain a best-fit dose response curve with the maximal response (E_{max}), the maximum agonist-induced response, and EC_{50}, the negative logarithm of drug concentration that yielded 50% of E_{max}, values determined. The differences in responses among the different groups were analyzed for statistical significance using two-tailed Student's t-test (Microsoft Excel, Microsoft Corp., USA) and two way analysis of variance (ANOVA, using GraphPad Prism ver.5.01) for unpaired observations. In all the cases differences were considered significant only if the P value less than 0.05 (P<0.05).

RESULTS

Effect of APCE on SBP measurements in conscious SH rats

Following 4 weeks of chronic treatment, no significant differences were observed in MABP between vehicle (Tween-80)-treated and untreated SH rats (P>0.05). Whereas the mean MABP values were significantly lower in the low, medium and higher doses (25, 50 mg/kg and 100 mg/kg respectively) of APCE and Verapamil-treated animals (P<0.0001) as compared to the vehicle-treated SH rats (Table 1).

Table 1: Effect of Systolic Blood Pressure (SBP) of chloroform extract of AP on various treatment groups on SHR rats

Treatment group	*SBP (mm Hg)*
Vehicle (Tween 80)	159.0±5.80
Chloroform extract of AP 25 mg/kg	147.4±4.58 *
Chloroform extract of AP 50 mg/kg	133.6±3.30***
Chloroform extract of AP 100 mg/kg	125.6±4.64***
Verapamil hydrochloride 12.5 mg/kg	130.7±3.63***

Values represent mean ± SEM of 6 experiments.
* P< 0.05; *** P < 0.001, treatment versus control group.

Effect of APCE on vascular functions *in vitro*

Endothelium-dependent relaxations to ACh

Figure 1 depicts the relaxation responses to acetylcholine (ACh), an endothelium-dependent vasodilator in aortas obtained from different treated groups of SH rats. The sensitivity (EC_{50}) and percentage maximal relaxation responses (R_{max}) were found to be highly significant (P<0.001) in 25 mg/kg (EC_{50} 7.83±0.03, R_{max} 63.17±0.59%), 50 mg/kg (EC_{50} 7.64±0.05, R_{max} 83.13±1.01%), 100 mg/kg APCE (EC_{50} 7.74±0.07, R_{max} 90.17±1.17%) and Verapamil 12.5 mg/kg (EC_{50}7.70±0.079, R_{max} 91.91±1.71%) when compared to vehicle (pEC_{50} 7.75±0.06, R_{max} 40.97±0.75%) treated group of animals.

Relaxations to Sodium Nitroprusside

Relaxation responses to SNP, an endothelium-independent vasodilator, were examined in aortic preparations obtained from various treatment groups (Fig. 2). The sensitivity and percentage maximal relaxation to SNP were highly significant (P<0.001) in APCE 25, 50, 100 & Verapamil 12.5 mg/kg treated SH rats. (EC_{50} 9.95±0.06, R_{max} 90.34±1.76; and EC_{50} 10.49±0.06, R_{max} 93.54±1.53% ; EC_{50} 11.04±0.06, R_{max} 104.2±1.42; and EC_{50} 10.90±0.063, R_{max} 105.0±1.41%) compared to control and vehicle (Tween 80) treated (EC_{50} 9.19±0.06, R_{max}75.01±1.7; EC_{50} 7.46±0.08, R_{max} 76.80±2.06). SH rats.

Contractions to high K^+ and phenylepherine (PE)

The aortic contraction responses to high K^+ (80 mM) and PE (1 µM) were measured in grams in aortas from the various treatment groups. No significant differences in the vasoconstrictor responses to both PE and high K^+ were seen between the vehicle treated-aortic rings and the control untreated SH animal's aortic rings. Whereas in aortas isolated from other treatment groups, the maximal tension to both PE and high K^+ remained comparable to that seen in the untreated animals (PE vs. APCE 25 mg/kg 0.38±0.02, 50 mg/kg 0.35±0.01, 100 mg/kg 0.31±0.01 and Verapamil 0.32±0.01; high K^+ vs. APCE 25 mg/kg 0.36±0.04, 50 mg/kg 0.29±0.014, 100 mg/kg 0.28±0.01 and Verapamil 0.30±0.03).

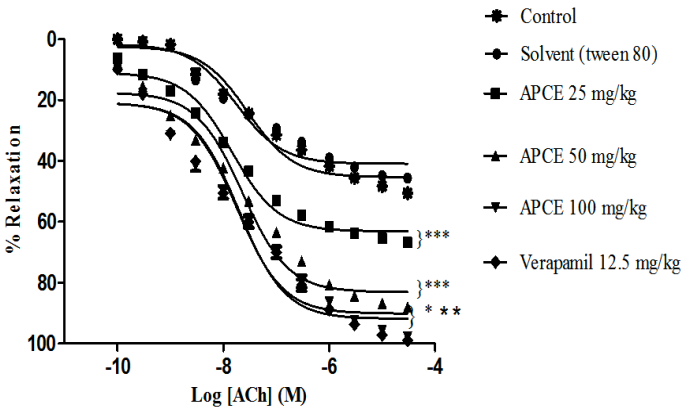

Figure 1: The effect of chloroform extract of AP (APCE) on acetylcholine-induced relaxation of epithelium intact SH rat aorta pre-contraction with 1µM phenylephrine. Symbols represent mean ± SEM of 8 experiments *** P < 0.001, Treatment versus control group.

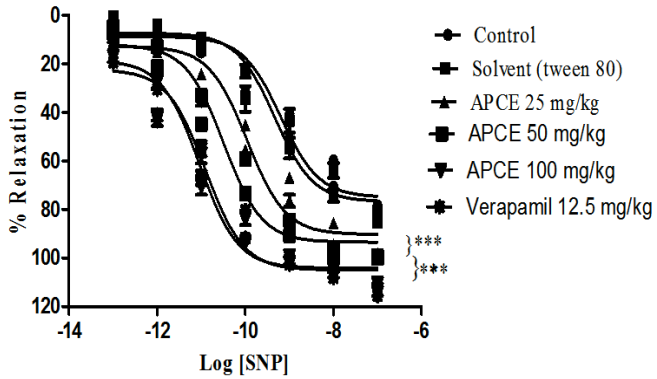

Figure 2: The effect of APCE on sodium nitroprusside-induced relaxation of endothelium denuded SH rat aorta pre-contracted with 1 µM phenylephrine. Symbols represent mean ± SEM of 8 experiments *** $P < 0.001$, Treatment versus control group.

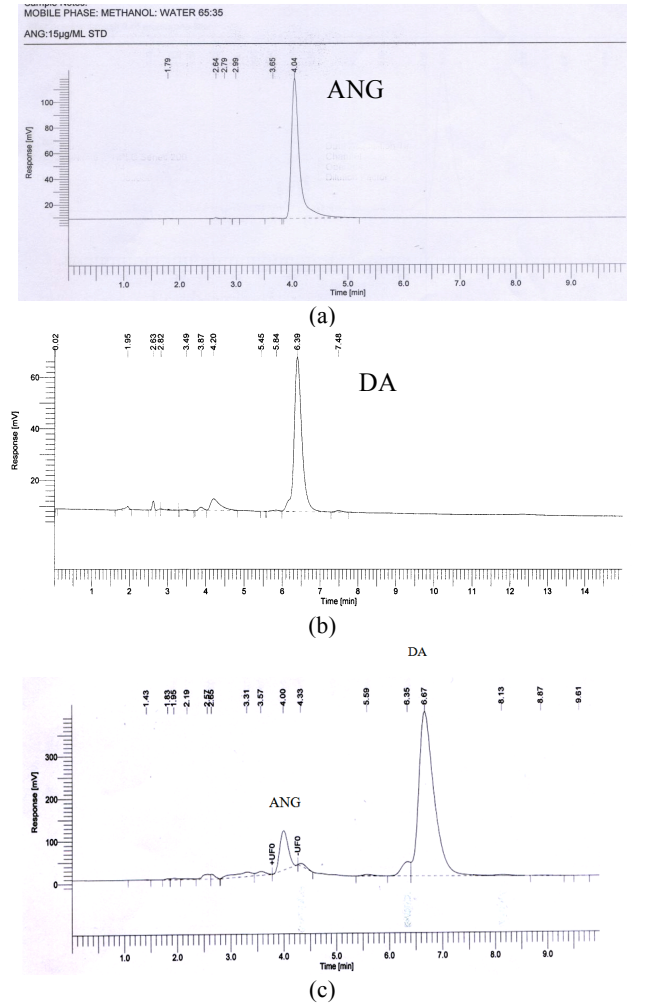

Figure 3. (A) HPLC chromatogram of the standard andrographolide (ANG), 14-deoxy-andrographolide (DA) and (c) *Andrographis paniculata* chloroform extract (APCE). HPLC was performed in a Shimadzu LC-10AT model using Nucleosil (Phenomenex) 5-µm C18 column (250 x 4.6 mm). Mobile phase was MeOH and H_2O (65:35) at a flow rate of 1 mL/min and injection volume 20 µL with SPD-10A UV detector set at 223 nm.

DISCUSSION

The diterpenoid lactone such as DDA from *Andrographis paniculata*, is reported to have hypotensive activity, by acting through β -adrenoceptors, autonomic ganglion receptor and angiotensin converting enzyme (ACE) inhibitory activity (Tan *et al*., 1998). The HPLC analysis of ANG and DA was carried out by following the method of Kumaran *et al*. (2003) using a simple isocratic elution technique. This method is simple, reproducible, and can be easily applied as a measure of quality control of APCE extracts for routine standardization, semi-quantitative analysis, and for optimization of extraction technique. The HPLC quantitative determination of both standard ANG, DA and extract sample eluted at the same retention time under identical conditions the amount of DDA in the extract sample with respect to the standard ANG and DA was found to be 19.7 mg/g, which was comparable with recent findings (Akowuah *et al*. 2006; Subramanian *et al*., 2006).

The present study clearly demonstrated that a 4-week APCE treatment in SH rats significantly increases the relaxation responses to ACh as a result of possible improvement in the endothelial function, these findings are consistent and comparable with the previous study by Tan *et al*., (1998). Conversely APCE possesses a remarkable capability to challenge the nor-epinephrine induced contractions resulting in vasorelaxation in isolated rat aorta (Naidu *et al*., 2007). The improvement in relaxation responses to ACh following chronic administration of APCE is most likely due to the activation of NO synthase and ultimate stimulation of NO production in endothelial cells. Moreover, the effects of chronic APCE administration are evidently suggestive of increased responsiveness of the vascular smooth muscle to NO since 4-week treatment with the extract was found to enhance the relaxation responses to the action of the endothelium-independent vasodilator SNP.

The standard drug Verapamil-treated group has also been observed to improve the relaxation responses to ACh. Similar study by Karaki *et al*., (1984a) demonstrated that in both rabbit and rat aorta the increment in response to high K^+ was specifically inhibited by Verapamil. In rabbit aorta, there seems to be two types of Ca^{2+} channels, one is activated by high K^+ and inhibited by Verapamil, while the other is activated by noradrenaline and indirectly inhibited by SNP. In rat aorta, both K^+ and noradrenaline-activated Ca^{2+} pathways are sensitive to both Verapamil and SNP.

In addition to the relaxation responses to ACh and SNP, 4 weeks treatment with APCE significantly reduced the mean SBP probably through its vasodilatory actions. In *vitro* exposure to high concentrations of Tween-80 may acutely alter endothelial cell and vascular smooth muscle function (Uluoglu *et al*., 1996). In this experiment, APCE was suspended with (10%, v/v) Tween-80. Chronic administration of low concentration of Tween-80 was without any detrimental effect on the endothelium and vascular smooth muscle since ACh- and SNP-induced vasorelaxation remained similar to those seen in the untreated animals.

Finally, endothelial protective effects of APCE were comparable to the effects of Verapamil, which acts by blocking the L-type Ca^{2+} current and high K^{+} activated pathways to relax the smooth muscle (Karaki *et al*., 1984b). In conclusion, chronic treatment with APCE significantly contributed to the improvement and alleviation of endothelial dysfunction in SH rats.

CONFLICT OF INTEREST

We have no financial, consultant, institutional and other relationships that might lead to bias or a conflict of interest.

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