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The Effect of Extracts of *Andrographis paniculata* Aerial Parts on Rat Thoracic Aorta

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ABSTRACT
Andrographis paniculata (AP), (Burf.) Nees. of *Acanthaceae*, has been used for centuries in Asia to treat GI tract and upper respiratory tract infections, fever, herpes, sore throat, and a variety of other chronic and infectious diseases. AP has cardio protective property and is familiarly known as "King of Bitters". The present study was aimed to investigate the vasorelaxant effect of different solvent extracts of AP on rat thoracic aorta. Petroleum ether, chloroform, and methanol extracts of AP are used in this study. Experiments are performed on male Sprague-Dawley (SD) rats for possible vasorelaxing activity of AP. Cumulative dose response curves are recorded by using isometric force displacement transducer Model FT-03 and it is connected to Grass Polygraph Model 79D. Additionally, HPLC study of chloroform extract of AP is conducted and compared with commercially available standard andrographolide. Petroleum ether and chloroform extract of AP is first suspended in 1% (v/v) DMSO then volume made up with Krebs solution. The average of responses to each concentration of the agonist is plotted on the ordinate the logarithm of the concentration of the agonist on the abscissa. Among all these extracts of AP, chloroform extract 80 and 160 µg/mL is found to be the highly significant ($P<0.001$) vasorelaxant effect on norepinephrine induced contraction on rat thoracic aortic ring preparations.
KEYWORDS: *Andrographis paniculata*, norepinephrine, rat aorta, vasorelaxant.

INTRODUCTION

Andrographis paniculata (AP), also commonly known as "King of Bitters," is a member of the plant family *Acanthaceae*, and has been used for centuries in Asia to treat gastrointestinal tract infection, upper respiratory infections, fever, herpes, sore throat, variety of other chronic and infectious diseases. The plant has no proven side effects, and it is beneficial in treating cardiovascular diseases (1).

Clot-dissolving drugs used in the emergency treatment of heart attacks appear to be as effective as angioplasty and may prevent some of the heart attacks or strokes that occur within one month of angioplasty. There is a delicate balance between the clotting, necessary to achieve healing and processes that will cause abnormal and unwanted clotting. It has been demonstrated that extracts of AP can increase the time required for blood clots to form, thus decreasing the risk of subsequent closing of blood vessels (restenosis) seen after angioplasty procedures (2). In studies done on rabbits by conducting angioplasty, AP extracts were shown significant prevent constriction of blood vessels (3). The rabbits received AP for three days before angioplasty and for four weeks after surgery. While, the arterial narrowing occurred in 100 % of animals not given AP, only 70% of those receiving AP showed narrowing. Narrowing caused by injury to the inner lining of the blood vessel and by high cholesterol in the diet was also found to be decreased

by AP. It appears that AP may be quite effective in preventing repeated narrowing of vessels after coronary angioplasty (4-5).

MATERIALS AND METHODS

Plant material

Fresh green aerial parts of the plant *Andrographis paniculata* were obtained from the cultivated nurseries of Malaysian Agriculture Development Institute (MARDI), Kelantan. The aerial parts of the plants were dried in the oven at a temperature of 38 °C for five days under shade and milled into coarse powder to increase the surface area.

Preparation of the extract

Successive extraction was done to get different extracts. The dried powdered aerial parts of *Andrographis paniculata* (883 g) was packed into a separate cellulose thimble and covered with cotton wool. The thimble was placed in a Soxhlet-extractor fitted with a quick-fit water condenser on the top and at the bottom 5 L round bottom distillation flask is attached. The distillation flask contained 2.5 L of the solvent required for extraction and a few anti-bumping granules were added. The dried powered leaves were first extracted with petroleum ether (60-80 °C) for a period of 48 h, after which the extract is collected and filtered and the marc left out was dried. Then the marc is extracted with chloroform for a period of 72 h and then again the same above procedure was repeated finally the left out marc was extracted with methanol for 72 hours. All the solvents containing extracts were evaporated to dryness using rotary evaporator (Buchi Labortechnik, Flawil, Switzerland) and dried in oven for further pharmacological evaluation.

Experimental animals

Experiments were performed on male Sprague-Dawley (SD) rats weighing between 220-260 g. The animals were bred and housed in the animal house, School of Pharmaceutical Sciences, Universiti Sains Malaysia. The animals were housed in propylene cages along with commercial saw dust as bedding material and allowed to free access of food (normal laboratory chow, Gold Coin ®, Penang, Malaysia.) and tap water *ad libitum*. All the animal experiments were approved by the Animal Ethics Committee, Universiti Sains Malaysia.

Organ baths

The organ bath consists of an inner jacket with an inlet and outlet of physiological salt solutions and a tissue holder with attached plastic tubes at the base to allow aeration and covered with outer warming jacket maintained at 37 °C by the temperature controlled circulating water bath. The tissue (organ) bath used was 10 mL.

Chemicals and Drugs

Norepinephrine, ascorbic acid and andrographolide were purchased from (Sigma Aldrich Chemical Co, USA). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), magnesium sulphate ($MgSO_4$), glucose, petroleum ether, chloroform and methanol from R & M Chemicals, Essex, UK. All the chemicals were analytical grade.

Experimental studies

Preparation and setting up of rat aorta

The male Sprague-Dawley (SD) rats were sacrificed by cervical dislocation and exanguinated. The midline incision was made from the neck region down to the abdomen to expose the visceral content and abdominal aorta. The aorta was quickly removed and placed in a Petri dish containing Krebs' physiological salt solution with the following composition: NaCl (118.2 mM/L), KCl (4.7 mM/L), and $CaCl_2$. $2H_2O$ (2.5 mM/L), KH_2PO_4 (1.2 mM/L), $MgSO_4$ (1.1 mM/L), glucose (11.7 mM/L), $NaHCO_3$ (25.0 mM/L). The aorta was cleared from fatty and connective. All dissecting procedures were carried out with extreme care to protect from inadvertent damage to the vessel.

Record of isometric vascular tone

The aortic rings were suspended in a tissue bath containing Krebs' physiological salt solution (KPSS) (pH 7.4) at 37 °C, while being continuously bubbled the preparation with gas mixture of (95 %) O_2 and (5 %) CO_2. The initial tension placed on the aortic rings was 1.0 g, and the changes in isometric tension were recorded using a force-displacement transducer (Grass FT 03, Quincy, MA USA) connected to a Grass polygraph recording system (Model 7E). In the first set of experiments, the aortic rings were induced to contract with norepinephrine starting with low concentration to high concentration that produce maximum contraction (1×10^{-12} M to 3×10^{-5} M) to obtain a dose response curve. Once the maximum response to norephenephrine (NE) had been obtained, the aortic rings were washed every 20 min with KPSS until the tension returned to the basal level. Predetermine concentration of extracts were incubated for 20 min in the organ bath and then a new dose response curve of norepinephrine was obtained. In the second experiment, the first set of aortic rings were induced to contract with phenylephrine (3×10^{-6} M). Once the maximal response to phenylephrine had been obtained, the aortic rings were washed every 20 min with KPSS

until the tension returned to the basal level. The rings were then exposed to phenylephrine (3×10^{-6}M) for 30 min, and then aortic relaxation was carried out by cumulative addition of extracts of medicinal plants. The vehicle, 1 % (v/v) dimethylsulfoxide (DMSO) used to dissolve the extract was devoid of any effects with norepinephrine induced contraction. After each test, the aortic rings should be washed three times with fresh KPSS and allowed for 30 to 40 minutes to equilibrate.

Calculation of responses

The largest contraction induced by agonist (norepinephrine as α-agonist) was taken as the maximum response. The response of aorta in the presence of different concentration of extracts is expressed as a percentage of maximum response (100%). The responses of 6-8 tissues were recorded. The average of responses to each concentration of the agonist is plotted on the ordinate and the logarithm of the concentration of the agonist on the abscissa. The E_{max} and EC_{50} (negative logarithm of drug concentration that yielded 50 % of E_{max}) 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} is the maximum agonist induced response) and EC_{50} values determined.

High Performance Liquid Chromatography (HPLC) analysis

HPLC-UV analysis was performed on a Shimadzu LC-10AT (Shimadzu Corporation, Kyoto, Japan) system 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) 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. HPLC-grade methanol (JT Baker Co, USA) was used for this purpose. The analysis was run at a flow rate of 1 mL/min. The detection was set at a wavelength of 223 nm. Stock solution was prepared at a concentration of 1 mg/mL in mobile phase. The stock solution was 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 10 % (v/v) APCE was prepared by dissolving 10 mg of 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 standard and extract samples were injected in triplicates in a sample volume of 20 µL. Before injection, the extract samples were filtered through a 0.2 µm polytetrafluoroethylene (PTFE) membrane (Aervent ® Disposable Filters, Millipore Corporation, MA, USA).

Statistical analysis

The results were analyzed by sigmoidal non-linear regression of concentration response curves. All data were expressed as means ± S.E.M (n=8). The significance difference was evaluated by using repeated measures of two-way analysis of variance (ANOVA) followed by Bonferroni Multiple Comparison test were applied to compare between groups using GraphPad software, 5.0.1, USA. All the cases statistical differences were considered significant only if the 'P' value were less than 0.001 ($p<0.001$).

RESULTS

The effect of petroleum ether (PE) extract of Andrographis paniculata on norepinephrene-induced contraction of isolated aortic strip preparations

It was found that the cumulative dose response curve of norephenephrine induced contraction of rat aortic strip preparation was not significantly reduced by incubation in the 0.25 mg/mL ($P>0.05$) of petroleum ether extract of *A.paniculata* (Figure 1). EC_{50} values for control, DMSO and 0.25mg/mL as follows (7.249 ± 0.05, 6.73 ± 0.05, and 7.17 ± 0.05 %). In contrast, norepinephrine induced contraction of isolated aortic strip preparation were significantly inhibited ($P<0.001$) in presence of 0.5, 1.0 and 2.0 mg/mL of petroleum ether extract of *A.paniculata.* Petroleum ether extract pretreated (EC_{50}: 7.175 ± 0.05, 6.55 ± 0.07 and 6.28 ± 0.08) and (% R_{max}) values (74.29 ± 2.16, 63.99 ± 1.91 and 30.48 ± 1.20), rat aortic rings.

The effect of methanolic extract of Andrographis paniculata (AP) on norephenephrine -induced contraction of isolated aortic strip preparations

The cumulative dose response curve of norephenephrine was dose dependently inhibited by methanolic extracts showed concentration dependent inhibition in normal, DMSO, 0.25, 0.5, 1.0 and 2mg/mL incubated SD (EC_{50}: 7.25 ± 0.05, 7.04 ± 0.05, 7.62 ± 0.07, 7.34 ± 0.067, 7.44 ± 0.77 and 6.06 ± 0.06) rat aortic rings. In contrast the maximal percentage (R_{max}) of vasoconstriction in normal, vehicle (DMSO) 0.1 %, 0.25, 0.5, 1.0 and 2.0 mg/mL incubated ($R_{max:}$ 97.67 ± 1.68 %, 88.14 ± 1.85 %, 82.44 ± 2.04 %, 65.14 ± 1.52 %, 39.05 ± 1.45 % and 24.32 ± 0.82 %) aortic rings. Only 0.5, 1.0 and 2.0 mg/mL methanolic extract incubated

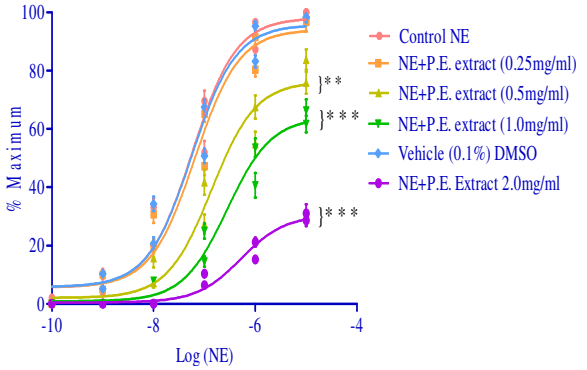

*Figure 1 : Contractile responses to norephenephrine (NE) of aortic rings in the presence of petroleum ether extract (P.E. Extract) 0.25, 0.5 and 1.0 mg/mL (n=8) **p<0.001, *** p<0.001*

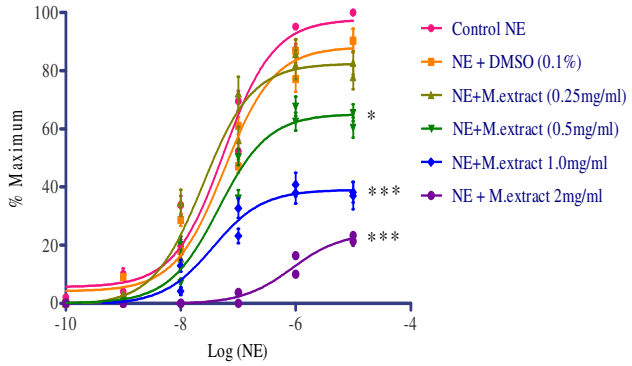

*Figure 2 : Contractile responses to norephenephrine (NE) of aortic rings in the presence of methanol extract (M. Extract) 0.25, 0.5, 1.0 and 2.0 mg/mL (n=8) * p<0.05, ** P<0.01 and *** P< 0.001*

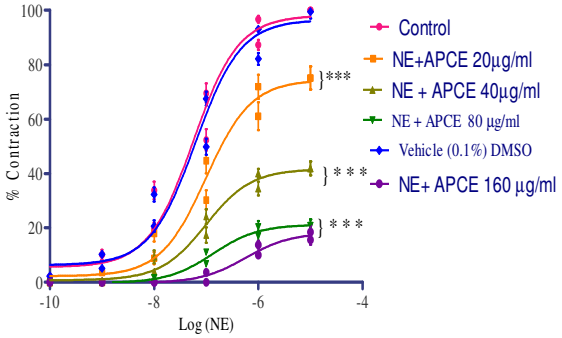

*Figure 3 : Contractile responses to norephenephrine (NE) of aortic rings in the presence of Andrographis paniculata chloroform extract (APCE) 20, 40, 80 and 160 µg/mL (n=8) *** p<0.001*

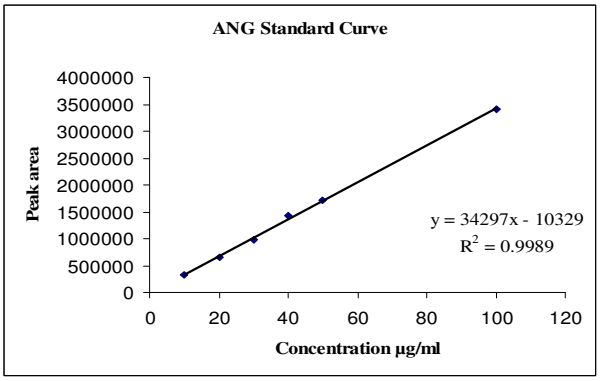

Figure 4. Standard calibration curve of ANG

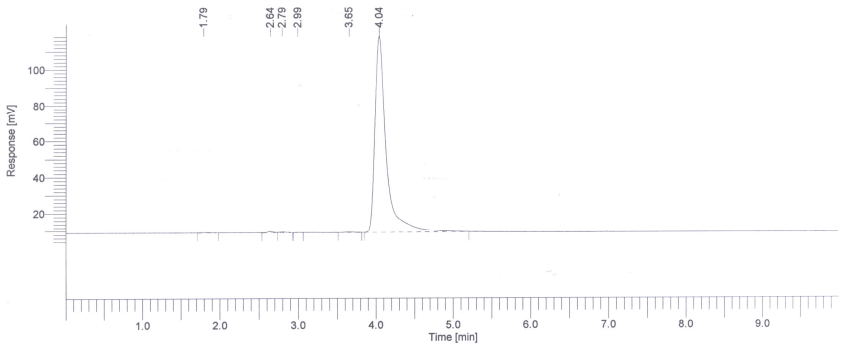

Figure. 5. A representative chromatogram of pure commercial ANG

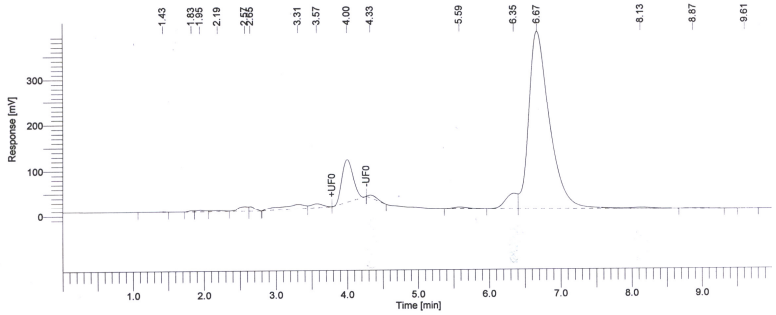

Figure 6. A representative chromatogram of Andrographis paniculata chloroform extracts (APCE)

aortic rings were significantly challenged the norepinephrine induced contractions (Figure 2).

The effect of chloroform extract of AP on norepinephrine-induced contraction of isolated aortic strip preparations

Pretreatment with chloroform extract of *Andrographis paniculata* reduced the maximal norepinephrine induced contraction in rat aortic rings from normal Sprague Dawely rats (SD rats). (R_{max} = 98.07 ± 1.69 %, EC_{50} = 7.249 ± 0.05) (Figure 3), DMSO (R_{max} = 99.58 ± 1.99%, EC_{50} = 7.17 ± 0.05), chloroform extract 25 µg/mL (R_{max} = 74.38 ± 2.10 %, EC_{50} = 7.0 ± 0.07), 40 µg/mL (R_{max} = 41.63 ± 1.19 %, EC_{50} = 7.0 ± 0.07), 80 µg/mL (R_{max} = 21.25 ± 0.79 %, EC_{50} = 6.87 ± 0.09), and 160 µg/mL (R_{max} = 18.20 ± 0.9 %, EC_{50} = 6.24 ± 0.09) rats.

HPLC analysis

Linearity

The calibration curve demonstrated good linearity and correlation coefficient over the given 10-100 µg/ml. the mean linear regression equation from six replicate curve was y = mx + c with a correlation coefficient of 0.9996. The calibration curve is showed in Figure 4.

Accuracy, precision and limit of detection

Both Intra-day and inter-day accuracy and precision was given by relative error (% RE) and relative standard deviation (% R.S.D). The intra-day accuracy and precision of ANG were ≤ 1.74 and ≤ 3.46. The inter-day accuracy and precision ANG were ≤ 0.58 and ≤ 2.29. The limit of detection was found to be (10 µg/ml) ANG was found to be ≤ 0.13 and ≤ 2.17. The inter-day accuracy and precision for limit of quantification for ANG was found to be ≤ 1.94 respectively.

DISCUSSION

The present study shows that petroleum ether, chloroform and methanolic extracts of *Andrographis paniculata* produce concentration-dependent relaxation of aortic rings pre-contracted by norepinephrine. The EC_{50} and R_{max} values of these extracts suggest that the chloroform extract of *Andrographis paniculata* was more potent when compared to petroleum ether and methanolic extract of AP. The dose dependent contraction inhibition effects of AP were reversible, indicating that it does not cause tissue damage or tissue tolerance.

The phytochemical constituents of AP that have been identified include the diterpene lactones (6) and flavonoids (7-8). The *Andrographis paniculata* chloroform extract (APCE) inhibited in a concentration-dependent manner the contractile response induced by the norephenephrine in rat thoracic aorta. The presence of diterpene lactones and flavonoids in the extracts could significantly contribute for its vasorelaxant activity.

HPLC determination of the commercially available pure ANG was performed with a Nucleosil C18 (ODS) column

and UV detection was done at 223 nm with mobile phase of methanol: water, 65:35 (v/v). Earlier method (10) described a method using a mobile phase of chloroform: methanol (9:1, v/v) and detection at 254 nm. Hence, this method is a modification and was attempted because of the strong peak and better resolution of the compound at 223 nm. This was also confirm from λ_{max} of 223 nm obtained from absorption scan results of ANG.

Andrographolide (ANG) exhibited good linearity in the range from 10-100 µg/ml in the calibration curves with a correlation coefficient of 0.999. The LOQ of ANG was found to be 10 µg/ml within acceptable limits of accuracy and precision. Both intra-day and inter-day accuracy and precision were carried out at three different concentrations and were found to be within limits. The validation result shows that the developed HPLC method is specific, sensitive, and within acceptable accuracy and precision. Hence this method can be applied for routine analysis of ANG and can be used in routine quality control of *Andrographis paniculata* and standardization purposes by small scale local herbal industries in plant extracts. The system suitability parameters were found to be within acceptable limits. The ANG, peak was identified in the APCE by comparison of the retention time of commercially available pure ANG with a retention time (R_t) of 4.0 min, (Figure 5 and 6) under similar chromatographic conditions.

In conclusion, the results of this study indicated that chloroform extract of *Andrographis paniculata* shows a potent inhibitor of norepinephrine induced contraction on rat aorta. Further studies on mechanism involved in vasorelaxation are under investigation.

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