Vasorelaxant effect of diterpenoid lactones from *Andrographis paniculata* chloroform extract on rat aortic rings

R. N. Sriramaneni[1,3], Ameer Z. Omar[1], Salman M. Ibrahim[1], Sadikun Amirin[2], Asmawi Mohd Zaini[1]

[1]Department of Pharmacology, School of Pharmaceutical Sciences, Universiti Sains, Malaysia, [2]Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Universiti Sains Malaysia, [3]Island College of Technology, Department of Pharmacy, Sungai rusa, Balik pulau, Pulau Pinang, Malaysia

Submitted: 15-05-2010 Revised: 26-06-2010 Published: 07-09-2010

ABSTRACT

Background: The aim of the present study is to evaluate the possible mechanism of the vasorelaxant effect of the *Andrographis paniculata* chloroform extract (APCE) and diterpenoids, such as, 14-deoxyandrographolide (DA) and 14-deoxy-11, 12-didehydroandrographolide (DDA), on rat aortic rings. **Methods:** DA and DDA (10 μM to 40 μM) induce relaxation in the aortic rings pre-contracted with KCl (80 mM). **Results:** The IC_{50} values are 40.47 ± 1.44 and 37.43 ± 1.41%, respectively, and this inhibition is antagonized by increasing the Ca^{2+} concentration in the Kreb's medium. The results indicate that APCE, DA, and DDA may have a calcium anatgonist property. APCE, DA, and DDA also relax norepinephrene (NE)-induced sustained contractions with IC_{50} values 41.63 ± 1.19, 49.22 ± 2.76, and 37.46 ± 1.41% and this relaxant effect is unaffected by the removal of the endothelium or by the presence of indomethacin and Nω-nitro-L-arginine (L-NAME). Moreover, DA and DDA inhibit the phasic and tonic contractions induced by NE in a concentration-dependent manner and show the most potent inhibition on phasic contraction ($P < 0.01$). **Conclusion:** This study shows that APCE, DA, and DDA pre-treatment presents a more potent inhibition compared to post-treatment, after the tension has reached a steady state. These results suggest that the vasorelaxation of APCE, DA, and DDA direct the inhibition of the calcium influx. The vasorelaxant effect is more active in the calcium independent pathway and more sensitive in the intial stage of contraction.

KEYWORDS: *Andrographis paniculata*, APCE, aorta, 14-deoxyandrographolide, 14-deoxy-11, 12-didehydroandrographolide, vasorelaxation.

INTRODUCTION

Andrographis paniculata (AP) Nees. (Acanthaceae) is a medicinal plant commonly used in India, China, and Southeast Asia for the treatment of a large variety of illnessees, which include meningitis, acute hepatitis, and many other acute inflammatory conditions.[1,2] The motivation of selecting this plant is due to its high profile biological activity. Andrographolide (ANG) is found in the whole plant, but is most concentrated in the leaves. It is a diterpene containing a gama lactone ring connected to the decalin ring system via an unsaturated C-2 moiety.[3] It has multiple pharmacological activities such as protozoacidal, antihepatotoxic, anti-HIV, immunostimulative, anticancer, hypoglycemic, and hypotensive activities.[4-15] A phase I trial of ANG in HIV-positive patients and normal volunteers indicated that ANG may inhibit HIV-induced cell cycle dysregulation, leading to a rise in $CD4^+$ lymphocyte levels in HIV-infected patients.[16] ANG and 14-deoxyandrographolide (DA) are active principles of the medicinal plant AP is used for the prevention and treatment of the common cold in Scandinavia, and is known to have an anti-inflammatory, antiviral, anti-thrombotic, hypotensive, and anti-atherosclerotic activity.[11] ANG has also been reported to exhibit a nitric oxide (NO) inhibitory property in endotoxin-stimulated macrophages.[17] Moreover, AP blocks the entry of extracellular calcium, by blocking voltage-operated calcium channels on the smooth muscle, hence, inhibiting the calcium influx.[18]

Nitric oxide is an intracellular and intercellular signaling molecule generated from L-arginine, catalyzed by a family of enzymes called nitric oxide synthases (NOS), which are

Address for correspondence:
Raghava Naidu Sriramaneni, 1003, Arlene Ct, Apt#9, Bloomington, 61701, Illinois, USA,
E-mail: sraghavanaidu@yahoo.com

DOI: 10.4103/0974-8490.69125

either constitutive (cNOS) or inducible (iNOS). A wide variety of biological effects are attributed to this molecule including neovascularization.[19,20] Vasodilatation by smooth muscles relaxation, mediated by NO, is a prerequisite for endothelial cells to enter the angiogenic cascade,[21] and it is reported that increased NOS activity correlates positively with the increase of vascular density and tumor growth.[22] Of late, is has been reported that together with the AP extract and its major component, ANG inhibit tumor-specific angiogenesis by regulating the production of various pro- and anti-angiogenic factors, such as, pro-inflammatory cytokine, nitric oxide, vascular endothelial growth factor (VEGF), interleukin-1β (IL-2), and tissue inhibitors of metalloproteinase-1 (TIMP-1).[24] The three active diterpenoids from AP, including the aqueous AP plant extracts, have also been reported to have an inhibitory effect on platelet aggregation *in vitro*. The results indicate that APCE, DA, and 14-deoxy-11, 12-didehydroandrographolide (DDA), significantly inhibit thrombin-induced platelet aggregation in a concentration (1 to 100 μM) and time-dependent manner, while neoandrographolide has little or no activity.[23]

The main purpose of the present study is to investigate the vasorelaxant effect of the *Andrographis paniculata* chloroform extract (APCE) on rat thoracic aorta.

MATERIALS AND METHODS

Design of the study

Norepinephrine bitratrate (NE), andrographolide (ANG), 14-deoxyandrographolide (DA), 14-deoxy 11, 12-didehydroandrographolide (DDA), acetylcholine, ethylene-glycol-bis-(β-aminoethylene)-N,N,N',N'-tetraacetic acid (EGTA), N^{ω}-nitro-L-arginine methyl ester (L-NAME), indomethacin, and andrographolide were purchased from Sigma (St. Louis, MO, USA). Tween-80 obtained from (R and M) chemicals. All other reagents were of analytical grade. DA and DDA were obtained from Professor M. Kuroyanagi, School of Pharmaceutical Sciences and University of Shizuoka, Japan. DA, DDA, and APCE were dissolved in DMSO. The other drugs were dissolved in distilled water and further dilution was made with 0.9% NaCl. DMSO at the maximal concentration of 0.2 % (v/v) did not show any effect on the contractions induced by NE and high K^+ (80 mM).

Preparation of the plant extracts

The fresh aerial parts of *Andrographis paniculata* were obtained from the cultivated nurseries of the Malaysian Agriculture Development Institute, Kelantan, Malaysia, and authenticated by the School of Biological Sciences, Universiti Sains Malaysia. The dried plant were powdered using a miller and successively extracted with petroleum ether (60 – 80ºC) for a period of 48 hours, chloroform for a period of 72 hours, and methanol for 72 hours, using the Soxhlet apparatus. The powdered plant material was allowed to dry before commencing a new extraction process using a different solvent. All extracts were evaporated to dryness using the Buchi Rotavapor (Switzerland) and subsequently freeze-dried.

Rat aortic ring preparations

All studies were approved by the Laboratories Institutional Animal Care and Use Committee of the School of Pharmaceutical Sciences and Universiti Sains, Malaysia. Male Sprague-Dawely (SD) rats weighing 210 g to 260 g were executed by decapitation, and the thoracic aortas were isolated carefully and immersed immediately in ice-cold physiological salt solution (PSS) of the following composition (mM): NaCl-136.9, KCl-5.4, $NaHCO_3$-23.8, glucose-5.5, $CaCl_2$-1.5, and $MgCl_2$-1.0. The fat and connective tissues were cleaned off the preparation and the aorta was cut into rings (2 to 3) mm in length.

The concentration of $CaCl_2$ was changed to 0.5 mM or 4.5 mM in some experiments. To investigate the influence of external Ca^{2+}, in some experiments, $CaCl_2$ was omitted and EGTA (1 mM) was added and APCE, DA, and DDA pre-treated in Ca^{2+}-free PSS for 10 minutes. In the endothelium-denuded aorta, the endothelium was removed by gently rubbing with a 22 G needle and the absence of acetylcholine-induced relaxation was taken as an indicator.[25]

Tension recording

The tension of the aorta rings was recorded isometrically via a force-displacement transducer (Grass FT 03, Quincy, MA USA) connected to a grass polygraph recording system (Model 7E). The aortic rings were placed in an organ chamber with PSS and aerated continuously with carbogen at 37°C. A passive tension of 1.0 g was initially applied to each aortic ring and the rings were allowed to equilibrate at 37°C for 60 to 90 minutes. Next (80 mM) high K^+-PSS, made by substituting NaCl in normal PSS with equimolar KCl, was repeatedly applied, in order to provide a reference force for standardizing the experimental values. To determine the relaxant effect, APCE, DA, DDA were cumulatively applied when the contractile tension induced by the stimulants reached a steady level, and the concentration required to induce 50% inhibition (IC_{50}) was calculated from the cumulative concentration–inhibition curves, by linear regression.

Statistical analysis

All data were expressed as the means ± SEM for the number of experiments indicated. Statistical analysis was performed using the GraphPad Prism version 5.01 (USA)

and the two-way ANOVA followed by the bonferroni test, for multiple comparisons. Student's t-test was used to evaluate the difference between two groups at the same time. $P < 0.05$ was regarded as significantly different.

RESULTS

In the Ca^{2+} free kreb's physiological salt solution containing (80 mM) K^+, an addition of Ca^{2+} caused a stepwise increase of contraction in the rat aorta. After pre-treatment (incubation) for 20 minutes, DA, DDA, and APCE inhibited this contraction in a concentration-dependent manner [Figures 1-3]. The EC_{50} values of DA, (10 μM, 20 μM, and 40 μM) are 6.86 ± 0.05, 6.66 ± 0.06, and 6.48 ± 0.07%; and for DDA EC_{50} they are 6.78 ± 0.05, 6.65 ± 0.06, and 6.43 ± 0.069%, respectively. The EC_{50} values for APCE (10 μg/mL, 20 μg/mL, 40 μg/mL, and 80 μg/mL) are 6.58 ± 0.059, 6.17 ± 0.053, 6.27 ± 0.068, and 6.26 ± 0.079%, respectively.

To investigate the effect of the pre-treatment of DA on the concentration–response curves for KCl and NE, DA was added to the bathing solution, and after incubation of 15 minutes, the concentration–response curve for KCl and NE, in the presence of DA (10 M, 20 M, and 40 M) was achieved by adding KCl (10 to 80) mM and NE (10^{-10} to 10^{-5}) M to the physiological salt solution cumulatively [Figures 4 and 5]. The result showed that 20 M and 40 M of DA shifted the concentration–response curves of KCl to the right in a nonparallel fashion, and depressed their maximal responses to 71.17 ± 5.76%, the EC_{50} values were 6.98 ± 0.21, 40.47 ± .44%, and 7.168 ± 0.10%, respectively [Figure 3]. The result showed that the DA also shifted the concentration–response curves of NE to the right in a nonparallel fashion, and depressed their maximal responses to 71.17 ± 5.76 and 40.47 ± 1.44% for 20 M and 40 M concentrations, respectively [Figure 4]. These results when compared with the above-mentioned results [Figure 2] indicated that the relaxant effect of DA is more potent on the pre-treatment occasion than on the post-treatment one.

The result showed that DDA also shifted the concentration–response curves of NE to the right in a nonparallel fashion, and depressed their maximal responses to 49.22 ± 2.76 and 37.46 ± 1.41% for 10 M, 20 M DDA, and for the 40 M concentration, it was 6.83 ± 0.93%, respectively. The IC_{50} values for 10 M, 20 M, and 40M DDA were 6.91 ± 0.14, 6.80 ± 0.09, and 6.41 ± 0.30%, respectively [Figure 6]. These results, when compared with the above-mentioned results [Figure 2] indicated that the relaxant effect of DDA was more potent on the pre-treatment occasion than that of APCE or DA.

Role of endothelium in APCE-induced relaxation

To elucidate the role of the endothelium in APCE-mediated relaxation in the rat thoracic aorta, the concentration-dependent responses to APCE were studied in the endothelium-intact and endothelium-denuded rings, pre-contracted with NE. There was no significant difference in the relaxation under either condition (data not shown).

The aortic rings were bathed in Ca^{2+}-free medium (PSS prepared with distilled water, where Ca^{2+} was replaced with EGTA) for 15 minutes and then NE 1 μM was added. When the transient phasic contraction (induced by intracellular Ca^{2+} release) reached a peak, $CaCl_2$ 1.5 mM was given rapidly, and the tonic contraction (evoked by extracellular Ca^{2+} influx) was recorded. The aortic rings were washed with PSS and incubated to equilibrium in Ca^{2+}-free medium, Guan *et al.* Next, the transient phasic and the tonic contractions induced by NE were tested again after incubation with DA and DDA (10 μM, 20 μM, and 40 μM) or DMSO for 15 minutes and the potencies of the chloroform extract, DA, and DDA were compared with the vehicle group. Diterpinoids inhibited both the phasic and tonic contractions in a concentration-dependent manner and showed a more potent inhibition on phasic contraction ($P < 0.01$) [Figures 7 and 8].

Influence of different factors on the relaxant effect of APCE, DA, and DDA

To determine the APCE, DA, and DDA relaxation effect signal pathway, we have used different factors, including L-NAME, an irreversible inhibitor of constitutive nitric oxide (NO) synthase (nNOS) and a reversible inhibitor of inducible NO synthase (iNOS), indomethacin, a cyclooxygenase (COX) inhibitor. After the rings were precontracted by high K^+ -PSS (80 mM), L-NAME (100 μM) and indomethacin (10 μM) were applied to the PSS for 15 minutes, respectively, and then DDA (10 to 40) M, was added cumulatively [Figure 6]. The results showed that the relaxant action of APCE, DA, and DDA persisted when different reagents were pre-incubated and there was no significant difference among the different factor groups and the control group (data not shown).

DISCUSSION

Diterpene lactones such as DA, DDA, and APCE relaxed the contraction induced by high K^+ (80 mM), phenylepherine (PE), and norepinephrine (NE) in a concentration-dependent manner in the rat thoracic aorta. The relaxant action of DA, DDA, and APCE persisted in the endothelium-denuded aorta, suggesting that the relaxant property of euxanthone is endothelium-independent. The relaxing pattern of diterpenoids and APCE on high K^+-

induced contractions was dependent on the extracellular Ca^{2+} concentration. The higher the extracellular Ca^{2+} concentration, the lower was the inhibition observed on high K^+-induced contractions. On the contrary, the lower the extracellular Ca^{2+} concentration, the greater was the inhibition obtained on high K^+-induced contractions.

The influx of external Ca^{2+} through specific Ca^{2+}-channels or Ca^{2+}-release from the internal stores, plays an important role in the excitation–contraction coupling of the smooth muscle. By acting on specific membrane receptors, NE induces Ca^{2+} influx through the receptor-operated channels causing a tonic contraction,[26] and stimulates the formation of inositol 1,4,5-triphosphate (IP_3), which binds to and opens specific IP3-receptor channels in the sarcoplasmic reticulum membrane and induces Ca^{2+} release causing phasic contraction.[27] On the other hand, the high K^+-induced contraction of the smooth muscle is the result of an increase in Ca^{2+} influx through the potential-dependent Ca^{2+} channels[28] and the potential-operated calcium channel activation is also involved in the NE-induced contraction. In the present experiments, DA, DDA, and APCE dose-dependently inhibited both the NE-induced tonic contraction and high K^+-induced contraction, suggesting that euxanthone acts as a Ca^{2+} channel blocker for both the receptor-operated and potential-dependent channels. We also found that DA, DDA, and APCE inhibited the NE-induced phasic contraction, suggesting that DA, DDA, and APCE inhibited the Ca^{2+} release from the sarcoplasmic reticulum.

The removal of the endothelium did not affect the log concentration-relaxing response curves of the cumulative DA, DDA, and APCE to high K^+ (80 mM)-induced precontraction, suggesting that the relaxant effect of euxanthone was endothelium-independent. The log concentration-relaxing response curve of the cumulative addition of DA, DDA, and APCE to high K^+ (80 mM)-induced precontraction was not affected by indomethacin (10 μM), a cyclooxygenase inhibitor, suggesting that its relaxant effect was not via prostagladin synthesis. L-NAME (100 μM) NO-synthase inhibitor did not affect the log-concentration relaxing response curve of DA, DDA, and APCE, suggesting that NO was not involved in the vasorelaxant effect of DA, DDA, and APCE.

To date, however, only a few studies have evaluated the effect of diterpenoids on *Andrographis paniculata* in calcium independent vascular contractions. The present results show that the vasorelaxation of DA, DDA, and APCE may be through the inhibition of the Ca^{2+} influx, whereas, the inhibitions of the intracellular calcium make a major contribution to the inhibitory effect of DA, DDA, and APCE on NE-induced phasic and tonic contractions, at lower concentrations. These results suggest that the vasorelaxation of diterpenoids (DA, DDA) and APCE may occur through multiple pathways involved in this calcium-independent pathway, which is the major pathway for the direct inhibition of calcium influx.

ACKNOWLEDGMENTS

This research study was supported by a grant from the Ministry of Science, Technology and Environment of Malaysia (MOSTE) (grant no. 304/PFARMAI/640043/KI05).

REFERENCES

1. Chopra R, Nayar SL, Chopra IC, editors. Glossary of Indian Medicinal Plants. Council for Scientific and Industrial Research New Delhi 1956.
2. Chang H, But P. Pharmacology and applications of Chinese material medica. In: Yeung SS, Yao SC, Wang LL, editors. Singapore: World Scientific; 1987.
3. Kleipool R. Constituents of *Andrographis paniculata*. Nature 1952;169:33-4.
4. Handa S, Sharma A. Hepatoprotective activity of andrographolide from *Andrographis paniculata* against carbon tetrachloride. In J Med Res 1990;92:276-83.
5. Siripong P, Konckathip B, Preechanukool K, Picha P, Tunsuwan K, Taylor WC. Cytotoxic diterpenoid constituents from *Andrographis paniculata* Nees leaves. Sci Soc Thai 1992;18:187-94.
6. Kapil A, Koul IB, Banerjee SK, Gupta BD. Antihepatotoxic effects of major diterpenoid constituents of *Andrographis paniculata*. Biochem Pharma 1993;46:182-5.
7. Visen P, Shukla B, Patnaik G, Dhawan B. Andrographolide protects rat hepatocytes against paracetamol-induced damage. Ethnopharmacology 1993;40:131-6.
8. Puri A, Saxena R, Saxena RP, Saxena KC, Srivastava V, Tandon JS. Immunostimulant agents from *Andrographis paniculata*. Nat Prod 1993;56:995-9.
9. Matsuda T, Kuroyanagi M, Sugiyama S, Umehara K, Ueno A, Nishi K. Cell differentiation-inducing diterpenes from *Andrographis paniculata* Nees, Chem Pharm Bull (Tokyo) 1994;42:1216-25.
10. Basak A, Cooper S, Andree G, Banik U, Michel C, Nabil G. Inhibition of proprotein convertases-1, -7 and furin by diterpines of *Andrographis paniculata* and their succinoyl esters. Biochemistry 1999;338:107-18.
11. Amroyan E, Gabrielian E, Panossian A, Wikman G, Wagner H. Inhibitory effect of andrographolide from *Andrographis paniculata* on PAF induced platelet aggregation. Phytomedicine 1999;10:47-60.
12. Zhang X, Tan B. Antihyperglycaemic and antioxidant properties of *Andrographis paniculata* in normal and diabetic rats. Clin Exp Pharm Phy 2000;27:358-63.
13. Stanslas J, Liew PS, Iftikhar N, Lee CP, Saad S, Lajis N, *et al*. APAG-1 as antitumour agent. European Cancer 2001;37:169.
14. Rajagopal S, Ajaya KR, Devi DS, Satyanarayana C, Rajagopalan R. Andrographolide, a potential cancer therapeutic agent isolated from *Andrographis paniculata*. Exp Ther Oncol 2003;47-158.
15. Srinivasa R, Hamzah AS, Saad MS, Lajis NH, Stevens

MFG, Stanslas J. Semisynthesis and cytotoxic activities of andrographolide analogues. J Enzyme Inh Med 2006;21:145-55.

16. Calabrese C, Berman S, Babish J, Ma XS, Dorr M, Wells K, *et al.* A phase I trial of andrographolide in HIV positive patients and normal volunteers. Photo Res 2000;14:333-8.
17. Chiou WF, Chen CF, Lin JJ. Mechanisms of suppression of inducible nitric oxide synthase (iNOS) expression in RAW 264.7 cells by andrographolide. Br J Pharmacol 2000;129:1553-60.
18. Burgos RA, Imilan M, Sanchez NS, Hancke J. *Andrographis paniculata* (Nees) selectively blocks voltage-operated calcium channels in rat vas deferens. J Ethnopharmacol 2000;71:115-21.
19. Josko J, Mazurek M. Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis. Med Sci Monit 2004;10:89-98.
20. Buttery L, Springall D, Andrade S, Riveros-Moreno V, Hart I, Piper PJ, *et al.* Induction of nitric oxide synthase in the neo-vasculature of experimental tumors in mice. J Pathol 1993;171:311-9.
21. Griffioenaw MG, Angiogenesis: Potentials for pharmacologic intervention in treatment of cancer, cardiovascular diseases and chronic inflammation. Pharmacol Rev 2000;52:237-68.
22. Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: Novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mole Cell Biol 2001;21:3995-4004.
23. Kuttan G, Sheeja K, Guruvayoorappan C. Antiangiogenic activity of *Andrographis paniculata* extract and andrographolide. Int Immunopharmacol 2006;7:211-21.
24. Thisoda, P, Rangkadilok, N, Pholphana, N, Worasuttayangkurn, L, Ruchirawat, S, Satayavivad J. Inhibitory effect of *Andrographis paniculata* extract and its active diterpenoids on platelet aggregation. Eur J Clin Pharmacol 2006;553:39-45.
25. Naidu S, Asmawi M, Amirin S. Vasorelaxant effect of chloroform extract of *Andrographis paniculata* on in-vitro rat thoracic aorta. Workshop on the "Isolated Tissue Preparations and HPLC Training" jointly organized by International Islamic University Malaysia and Malaysian Society of Pharmacology and Physiology, Kuantan, Malaysia. 2007.
26. Paoletti R, Govonis S. Classification of calcium antagonists: Proposal of the WHO committee. Pharmacol Res Commun 1994;19:195-204.
27. Broekaert A, Godfraind T. A comparison of the inhibitory effect of cinnarizine and papaverine on the noradrenaline and calcium-evoked contraction of isolated rabbit aorta and mesenteric arteries. Eur J Pharmacol 1979;**53**:281–8.
28. Van Breemen C, Farinas B, Gerba P, Mc Naughton E, Broekaert A, Godfraind T. Excitation–contraction coupling in rabbit aorta studied by the lanthanum method for measuring cellular calcium influx. Circ Res 1972;30:44-54.

Source of Support: Ministry of Science, Technology and Environment of Malaysia (MOSTE) (grant no. 304/PFARMAI/640043/KI05)., **Conflict of Interest:** None declared.