Effects of Phikud Navakot Extract on Vascular Reactivity in the Isolated Rat Aorta

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The aim of the present study is to investigate the effect of standardized Phikud Navakot extract (NVKE) on aortic rings from male Sprague Dawley rats. Changes in tension were measured using an isometric force transducer and recorded on the PowerLab recording system. Vasorelaxant effect of NVKE was examined in the presence of indomethacin (10 microM), N\textsuperscript{-}nitro L-arginine methyl ester (L-NAME, 300 microM), methoxamine (0.1-300 microM), carbachol (1 nanoM-30 microM), or sodium nitroprusside (0.1 nanoM-10 microM). The results showed that NVKE (1-300 microg/mL) caused vasorelaxation in a concentration-dependent manner with a pEC\textsubscript{50} value of 4.27 ± 0.24 and R\textsubscript{max} of 67.7 ± 13.9%. Pretreatment with indomethacin or L-NAME did not affect NVKE-induced vasorelaxation. However, co-incubation of indomethacin and L-NAME significantly reduced (p < 0.05) vasorelaxation to NVKE (100 microg/mL). Pre-treatment with NVKE significantly decreased (p < 0.05) endothelium-dependent relaxations to carbachol (R\textsubscript{max} = 52.90 ± 14.3%), but not to sodium nitroprusside. Moreover, contractions to methoxamine were unaffected after pretreatment with NVKE. The present study suggested that NVKE decreased vasorelaxation to carbachol in the rat aorta, which may exert at least against muscarinic receptors. These findings support the use of Phikud Navakot, a major ingredient of Yahom Navakot, against dizziness and fainting.

Keywords: Navakot, Aorta, Vasorelaxation, Angelica dahurica, Atractylodes lancea, Ligusticum chuanxiong, Angelica sinensis, Artemisia vulgaris, Saussurea costus, Picrorhiza kurrooa, Terminalia chebula, Nardostachys jatamansi

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Herbal medicines have become increasing popular worldwide as alternative medicines for treatment of numerous diseases including myocardial ischemia and atherosclerosis\textsuperscript{1,2}. Phikud Navakot (NVK) is a major ingredient of “Yahom Navakot”, a traditional Thai herbal formula used for treatment of cardiovascular symptoms included dizziness and fainting\textsuperscript{3}. Phikud Navakot is a set of nine herbs namely, “Kot Soa” (root of Angelica dahurica (Fisch.) Benth & Hook f., family Umbelliferae), “Kot Khamao” (rhizome of Atractylodes lancea (Thunb.) DC., family Compositae), “Kot Hua Bua” (root of Angelica dahurica (Fisch.) Benth & Hook f., family Umbelliferae), “Kot Khamao” (rhizome of Atractylodes lancea (Thunb.) DC., family Compositae), “Kot Chiang” (root of Angelica sinensis (Oliv.) Diels, family Umbelliferae), “Kot Chulalumpa” (aerial part of Artemisia vulgaris L., family Compositae), “Kot Kradook” (rhizome of Saussurea costus (Falc.) Lipsch., family Compositae), “Kot Kan-Prao” (rhizome of Picrorhiza kurrooa Royle ex Benth., family Scrophulariaceae), “Kot Pung Pla” (gall of Terminalia chebula Retz., family Combretaceae) and “Kot Jatamansi” (root and rhizome of Nardostachys jatamansi (D. Don) DC., family Valerianaceae). The actions of each Yahom recipient are still needed scientific supports. Likewise, a traditional Yahom powder containing S. costus (synonym S. lappa) increased diastolic blood pressure and mean arterial blood pressure, but had no effect on systolic blood pressure in healthy volunteers\textsuperscript{4}. L. chuanxiong and A. sinensis were used to increase blood supply to some organs including the heart in Chinese herbal medicine\textsuperscript{4}, and the extract from T. chebula also showed hypotensive effect\textsuperscript{5}.

In the present study, the ethanolic extract of NVK (NVKE) was prepared and its effect on vascular reactivity was herein reported for the first time. The possible mechanisms underlying the effect of NVKE on endothelium-intact rat thoracic aorta were also investigated.
Material and Method

Drugs and chemicals

Carbachol hydrochloride, methoxamine, indomethacin, N\textsuperscript{\textregistered}-nitro-L-arginine methyl ester (L-NAME) and sodium nitroprusside (SNP) were purchased from Sigma-Aldrich Chemical Co (St. Louis, USA). All other reagents were of analytical grade. Indomethacin was dissolved in absolute ethanol. The remaining drugs were dissolved in deionized water. All drugs were freshly prepared on the day of experimentation.

Preparation of extracts

Phikud Navakot was obtained from an equal amount of nine crude drugs as mentioned above. The materials were purchased from a traditional Thai pharmacy in Bangkok, Thailand, on April 2011 and identified by Dr. Uthai Sotanaphun. Their voucher specimens (NVK01-09) have been deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand. The NVK coarse powder was soaked in 10 times by weight of 80% ethanol for overnight and continuously extracted twice at 100\degree C for 3 h each. The two subsequent extracts were combined and concentrated under reduced pressure to give NVKE. The phenolic content of the extract was 15.17 ± 0.98% GAE (gallic acid equivalence) determined by Folin-Ciocalteau method\textsuperscript{[6]}. The extract solution was freshly prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO in organ bath was 0.27% (v/v).

Preparation of the rat aorta

Male Sprague Dawley rats (250-300 g) were supplied by the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed in a temperature-controlled room under 12-h light/dark cycle. They had free access to water and standard diet. All procedures were approved by the Animal Research Ethics Committee of the Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand (Approval No. 15/2553 and 12/2554).

The rats were anesthetized with 50 mg/kg Zoletil (tiletamine chloridrate and zolazepan chloridrate) by intramuscular injection into quadriceps muscle\textsuperscript{[7]}, and sacrificed by cervical dislocation. The thoracic aorta was dissected rapidly, carefully cleaned of adhering fat and connective tissue and cut into 5 mm ring segments. Each ring was transferred to a jacketed organ bath filled with 20 mL of modified Krebs-Henseleit (KH) solution containing (in mM): NaCl 118, KCl 4.7, MgSO\textsubscript{4} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.2, NaHCO\textsubscript{3} 25, CaCl\textsubscript{2} 2 and D-glucose 10. The bath solution was maintained at 37\degree C, and continuously bubbled with carbogen, the mixture of 95% O\textsubscript{2} and 5% CO\textsubscript{2}. The rings were mounted between two triangular stainless steel hooks: one hook was fixed to the organ bath and the other was connected to a force-displacement transducer. The bath solution was exchanged with pre-warmed oxygenated KH solution every 15 minutes. The tension was monitored with isometric force transducers (MLT 0210, New South Wales, Australia) and displayed on a PowerLab recording system (AD instruments, New South Wales, Australia) for data acquisition.

Experimental protocols

Prior to addition of drugs or the extract, tissues were equilibrated for 1 h, methoxamine (10-100 microM) was used to increase tone by approximately 1 g, then endothelium integrity was confirmed by the observation of more than 70% relaxation induced by 0.3 microM carbachol, an agonist of muscarinic cholinergic receptors. The buffer solution was replaced in order to retain baseline tension. Vasorelaxant effect was measured by cumulative concentration of NVKE. In vehicle-control experiments, DMSO alone was added at the same concentration as those used in the experiments with NVKE. The vasorelaxation response of aortic ring to NVKE (0.1-300 microg/mL) was performed without and with 30 min pre-incubation with the following inhibitors/activators: a cyclooxygenase (COX) inhibitor indomethacin (10 microM), or an endothelial nitric oxide synthase (eNOS) inhibitor L-NAME (300 microM) for 30 min before incubation of methoxamine (10-30 microM) required to induce equivalent levels of tone\textsuperscript{[8]}. Tissues were also incubated with 100 microg/mL NVKE for 30 min before the addition of an endothelium-dependent vasodilator (carbachol, 1-30 microM), an important vasodilator SNP (0.1 nanoM-10 microM) and a vasoconstrictor agent (methoxamine, 0.1-300 microM) to give cumulative concentration-response curves.

Statistical analysis

All data were expressed as mean ± SEM; n represents the number of observations, one for each animal. The concentration of vasorelaxant giving maximal smooth muscle relaxation (R\textsubscript{max}) and the EC\textsubscript{50} values (the concentration of test drug causing a half maximal relaxation of the aortic precontraction) were obtained from the concentration-response curve fitted
to a sigmoidal logistic equation using the GraphPad Prism package. \( R_{\text{max}} \) and pEC\(_{50} \) values (negative logarithm of the molar concentration of extract required to elicit 50% inhibition of maximum) between groups were compared by one-way analysis of variance (ANOVA) followed by Bonferroni’s post-hoc test. P-values less than 0.05 were considered statistically significant. In experiments to study the effects of inhibitors indomethacin and L-NAME, data did not fit to sigmoidal dose-response curves, the relaxant effects of NVKE were presented as the percentage reduction from the initial tone in each ring precontracted with methoxamine.

Results

**Vasorelaxant effect of NVKE on aortic ring**

The NVKE (0.1-300 microg/mL) induced a concentration-dependent relaxation in methoxamine-contracted aorta ring (Fig. 1) with the pEC\(_{50} \) value of 4.27 ± 0.24 and \( R_{\text{max}} \) of 67.7 ± 13.9%.

**Effects of L-NAME and indomethacin on action of NVKE**

To determine the mechanism of the vasorelaxation induced by NVKE (0.1-300 microg/mL), a NOS inhibitor L-NAME (300 microM) was used to determine whether NO-involved process. A non-selective COX inhibitor, indomethacin (10 microM) was also preincubated to exclude the vasorelaxant effect through the production of prostaglandin (PGI\(_2\))(7). Pretreatment with L-NAME or indomethacin alone did not affect the relaxant effect of NVKE. However, co-incubation of L-NAME and indomethacin significantly inhibited (p < 0.05) the percentage relaxation of tone of NVKE (100 microg/mL) from 51.64 ± 5.60 in control group to 22.60 ± 6.70 (Fig. 2).

**Effect of carbachol, sodium nitroprusside and methoxamine on action of NVKE**

To investigate whether NVKE act on the acetylcholine receptors by stimulating muscarinic receptors or alpha1-adrenoceptors of vascular smooth muscle, aortic rings were preincubated with NVKE (0.1-300 microg/mL) before cumulative addition of carbachol (1 nanoM-30 microM) or methoxamine (0.1-300 microM), respectively. A NO donor, SNP was also used to clarify the mechanism involved in endothelium-independent relaxation to NVKE in aortic rings.

The \( R_{\text{max}} \) of cumulative concentration response curves to carbachol was significantly inhibited (p < 0.05) by NVKE (100 microg/mL) as shown in Fig. 3. SNP (0.1 nanoM-10 microM) induced relaxation in a concentration-dependent manner in aortic ring. There was no significant difference of pEC\(_{50} \) and \( R_{\text{max}} \) between NVKE-treated and the control aortic ring (Fig. 4). However, the increased tone produced by
methoxamine after preincubation with NVKE (1-100 microg/mL) was not significant different from the control (Fig. 5).

Discussion
The present study investigated the effects of NVKE-induced vasorelaxation on endothelium-intact rat aorta via NO pathway, by blocking NOS and the COX pathway. The responsiveness of NVKE-incubated aortic ring was also studied using an endothelium-dependent vasodilator (carbachol, a muscarinic agonist), an endothelium-independent relaxation (sodium nitroprusside) and alpha1-adrenergic agonist (methoxamine).

Pretreatment with eNOS inhibitor L-NAME or COX inhibitor indomethacin alone did not affect the relaxation in NVKE-treated rat aortic rings, meanwhile coinubation of both inhibitors was able to decrease NVKE-induced vasorelaxation. These results suggested that the combined inhibitory effect of NOS and COX inhibitors may contribute as a synergistic role in NVKE-induced vasorelaxation of aorta as shown previously\(^9\). In addition, the opposite activity of its ingredients has been reported. In accordance with previous study, \textit{S. lappa} and its major active compounds, dehydrocostuslactone and costunolide, possessed vasorelaxant effect\(^1\). The cyclohexane and ethyl acetate extracts of \textit{A. dahurica} var. formosana as well as its major active ingredient, imperatorin, were shown to induce vasorelaxation via NO pathway\(^2\). In contrast, the ethyl acetate extract from \textit{A. Dahuricae}...
and gallic acid extracted from T. chebula inhibited lipopolysaccharide-stimulated macrophage in production of NO, COX2 and inducible NOS(12,13), which played an important physiological role of vasodilation. In the present study, the authors demonstrated that the $R_{\text{max}}$ to carbachol (1 nanoM-30 microM) was significantly attenuated in the presence of NVKE (100 microg/mL), indicating an antagonist effect of NVKE mediated mainly through muscarinic receptors as shown in mouse endothelium-intact aorta(14) as well as the smooth muscle cells and endothelial cells of rabbit thoracic aorta(15) and equine coronary artery(16). The antagonistic activity of NVKE on acetylcholine receptor may be useful for prevention or treatment against vasovagal syncope from various causes such as disease-related impairment of vascular adaptation to minor physiological stressors(17). In contrast, incubation of NVKE without inhibitors showed vasorelaxant activity to aorta, it is probably useful for protecting or improving the symptoms of cardiovascular diseases including coronary artery diseases(18). Meanwhile, the relaxation response to SNP of isolated aorta was the same in NVKE-treated and the control group. It may be concluded that the relaxation of NVKE-treated aorta is not involved in smooth muscle relaxation pathway(19) via activating soluble guanylyl cyclase(20). The vascular basal tone was generated by methoxamine as shown in previous study(7). The present study demonstrated that the contractions to methoxamine were not different between NVKE-treated and the control aortic rings. The results implied that NVKE-induced vasorelaxation was not mediated through inhibiting alpha1-adrenergic agonist(21) or did not affect Ca$^{2+}$-induced Ca$^{2+}$ release from sarcoplasmic reticulum and extracellular Ca$^{2+}$ influx(22).

To the authors knowledge, this is the first time showing that NVKE exerts mild vasorelaxant effects in concentration-dependent manner on rat aorta. NVKE was able to reduce carbachol-induced vasorelaxation in rat aorta, which at least in part through antagonism against muscarinic receptors.

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Potential conflicts of interest

None.

References

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ผลของสารสกัดพิกัดนวโกฐต่อการตอบสนองของหลอดเลือดเอออร์ตาที่แยกจากหนูขาว

พรรณี หนูชื่อดัง, อุทัย โสธนะพันธุ์, พัชรินทร์ เทพอารีนันท์

การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของสารสกัดมาตรฐานพิกัดนวโกฐต่อหลอดเลือดเอออร์ตาของหนูขาวเพศผู้สายพันธุ์ Sprague Dawley โดยวัดการเปลี่ยนแปลงแรงดันของหลอดเลือดควบคู่กับ isometric force transducer และบันทึกสัญญาณผ่าน PowerLab recording system. การศึกษาฤทธิ์คลายตัวของสารสกัดเอทานอลของพิกัดนวโกฐใช้ indomethacin (10 ไมโครกรัมต่อมิลลิลิตร) N-o-nitro L-arginine methyl ester (L-NAME) (300 ไมโครกรัมต่อมิลลิลิตร) methoxamine (0.1-300 ไมโครกรัมต่อมิลลิลิตร) carbachol (1 นาโนกรัมต่อมิลลิลิตร) หรือ sodium nitroprusside (0.1 นาโนกรัมต่อมิลลิลิตร) ผลการทดลองแสดงให้เห็นว่าสารสกัดพิกัดนวโกฐขนาด 1-300 ไมโครกรัมต่อมิลลิลิตร มีฤทธิ์คลายตัวในหลอดเลือดเอออร์ตาคลายตัวโดยความสามารถในการคลายตัว เท่ากับ 4.27 ± 0.24 และค่า Rmax เท่ากับ 67.7 ± 13.9% และพบว่าเมื่อบ่มเอออร์ตาด้วย indomethacin หรือ L-NAME ก่อนการบ่มด้วยสารสกัด ไม่มีผลต่อฤทธิ์คลายตัวของสารสกัดพิกัดนวโกฐ แต่เมื่อบ่ม indomethacin ร่วมกับ L-NAME มีผลยับยั้งฤทธิ์คลายตัวของหลอดเลือดที่ชักนำโดยสารสกัดพิกัดนวโกฐที่ความเข้มข้น 100 ไมโครกรัมต่อมิลลิลิตร ได้อย่างมีนัยสำคัญทางสถิติ (p < 0.05) นอกจากนี้สารสกัดพิกัดนวโกฐ (100 ไมโครกรัมต่อมิลลิลิตร) ยังลดฤทธิ์ของ carbachol ที่ทำให้เอออร์ตาคลายตัวอย่างมีนัยสำคัญทางสถิติ (p < 0.05) ที่ Rmax เท่ากับ 52.90 ± 14.3% ในขณะที่ไม่พบความแตกต่างเมื่อได้รับ sodium nitroprusside หรือ methoxamine จากผลการทดลองแสดงให้เห็นว่าสารสกัดเอทานอลของพิกัดนวโกฐมีฤทธิ์ต่อการคลายตัวของหลอดเลือดเอออร์ตาที่ชักนำโดย carbachol โดยเป็นไปอย่างมีนัยสำคัญทางตัวรับ muscarinic การศึกษาในครั้งนี้ช่วยสนับสนุนการใช้ประโยชน์จากสมุนไพรพิกัดนวโกฐ ซึ่งเป็นส่วนประกอบหลักของยาหอมนวโกฐในการทราบอาการหน้ามืดเป็นลม