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Tetra-acetylajugasterone a new constituent of *Vitex cienkowskii* with vasorelaxant activity

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ABSTRACT

Tetra-acetylajugasterone C (TAAC) was found to be one of the naturally occurring compounds of the Cameroonian medicinal plant *Vitex cienkowskii* which is responsible for a vasorelaxant activity of an extract of this plant. The evaluation of the underlying mechanisms for the relaxing effect of TAAC was determined using aortic rings of rats and mice. TAAC produced a concentration-dependent relaxation in rat artery rings pre-contracted with 1 μM noradrenaline (IC_{50}: 8.40 μM) or 60 mM KCl (IC_{50}: 36.30 μM). The nitric oxide synthase inhibitor L-NAME (100 μM) and the soluble guanylate cyclase inhibitor ODQ (10 μM) significantly attenuated the vasodilatory effect of TAAC. TAAC also exerted a relaxing effect in aorta of wild-type mice ($cGKI^{+/+}$; IC_{50} = 13.04 μM) but a weaker effect in aorta of mice lacking cGMP-dependent protein kinase I ($cGKI^{-/-}$; IC_{50} = 36.12 μM). The involvement of calcium channels was studied in rings pre-incubated in calcium-free buffer and primed with 1 μM noradrenaline prior to addition of calcium to elicit contraction. TAAC (100 μM) completely inhibited the resulting calcium-induced vasoconstriction. The same concentration of TAAC showed a stronger effect on the tonic than on the phasic component of noradrenaline-induced contraction. This study shows that TAAC, a newly detected constituent of *Vitex cienkowskii* contributes to the relaxing effect of an extract of the plant. The effect is partially mediated by the involvement of the NO/cGMP pathway of the smooth muscle but additionally inhibition of calcium influx into the cell may play a role.

Introduction

The genus *Vitex* belonging to the family of Verbenaceae includes approximately 250 known species of trees and shrubs within tropical and sub-tropical regions, although few species may be found in temperate zones (Correa, 1926; Arbonnier, 2004). *V. cienkowskii* Kotschy & Peyritsch is a deciduous tree, prescribed by Cameroonian traditional healers as one of the most popular plants widely used in many disorders including cardiovascular diseases such as hypertension (Nkeng-Efouet, 1987).

Hypertension is a major risk factor for stroke, myocardial infarction as well as heart and kidney failure worldwide and also in Cameroon. The prevalence of hypertension in Cameroon is estimated to be about 19.1% in urban areas and 15.4% in rural areas (Fourcade et al., 2007).

For populations with poor economic resources herbal drugs as tea were widely used like *V. Cienkowskii* which possessed multivalent pharmacological activities (Arbonnier, 2004). *V. cienkowskii* is one of the plants used in Cameroon to cure cardiovascular disorders such as hypertension. Thus, pharmacological validation of medicinal plants for antihypertensive effects or evaluation of ethnomedical treatment methods could have great benefit.

Initial chemical characterization studies on *V. cienkowskii* have been carried out and led to the identification and isolation of a

Abbreviations: cGK(I), cGMP-dependent protein kinase (I); (e)NOS, (endothelial) nitric oxide synthase; L-NAME, Nω-nitro-L-arginine methyl ester; MMEvc, methylene chloride-methanol (1:1) extract of *V. cienkowskii*; NA, noradrenaline; NO, nitric oxide; ODQ, 1-H-[1,2,4]-oxadiazolo-[4,3a]-quinoxalin-1-one; ROC, receptor-operated ion channels; TAAC, tetra-acetylajugasterone C; TRP, transreceptor potential; VOC, voltage-operated ion channels.

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number of pentacyclic triterpenoids and ceramide which contribute to the vasorelaxation effect (Dongmo et al., 2011).

The activity of the plant extract provided strong evidence that there might be additional unidentified compounds contributing to the effect of the *V.cienkowskii* extract. Therefore, the present study was undertaken to isolate these compounds and to elucidate its mode of action by functional experiments.

Materials and methods

Plant material

The stem bark of *V. cienkowskii* was collected in Foumban, Cameroon, in May 2007 and identified at the National Herbarium, Yaoundé Cameroon, where a voucher specimen (number 32721HNC) is deposited for future reference.

Extraction and isolation

The air-dried stem bark of *V. cienkowskii* was ground to a fine powder. The stem bark powder (2.5 kg) was extracted by maceration at room temperature in 4 l of CH_2Cl_2–MeOH (1:1) for 48 h. The mixture was then filtered and the filtrate was evaporated in a rotary evaporator at 40 °C under reduced pressure, yielding 27.54 g of a brown powder. The residual was extracted by maceration with 4 l of MeOH for 48 h yielding 18 g of a brown powder.

Since CH_2Cl_2-MeOH and MeOH extracts showed almost equal activities in pharmacological experiments and similar TLC profiles after development in different solvent systems, we decided to put the two fractions together and elucidate its active principles. The bioassay-guided fractionation based on vasorelaxant activity was used for the isolation of active principles. A portion of 25 g of the extract was subjected to column chromatography using silica gel (0.063–0.200 mm). The column was eluted using the following solvent systems: hexane-EtOAc (1:0 → 0:1) followed by MeOH–EtOAc (0:1 → 1:0). 180 fractions (200 mL each) were collected and combined to produce 19 pooled fractions (F-I to F-XIX) based on their TLC profiles. According to an initial vasorelaxant test, only fractions F-VI, F-VII, F-VIII, F-X, F-XV and FXVI were active showing about 30% relaxation in noradrenaline-induced contraction of rat aorta. From the active fractions, F-XVI was resubmitted to column chromatography on silica gel (0.063–0.200 mm) and eluted with MeOH-EtOAc (6:4 → 3:7) to give 10 subfractions (f1–f10). Subfraction f6 was rechromatographed on silica gel (0.063–0.200 mm) and eluted with EtOAc-MeOH (4:6 → 8:2) to give the pure **compound 1**. Subfraction f8 was treated in the same way but eluted with EtOAc-MeOH (7:3 → 8.5:1.5) to yield the pure **compound 2**. The **compounds 1** and **2** were identified by interpretation of their spectral data (IR, NMR ^{1}H, ^{13}C) as well as by comparison with literature data.

Pharmacological investigations

Preparation of rat and mice aortic rings

Rats weighing 150–200 g from Charles River, Kisslegg, Germany were used. The animals were killed by decapitation in ether anaesthesia. The thoracic aorta was quickly removed, freed of connective tissue and placed in the bath chamber as described previously (Dongmo et al., 2011). The measurement signals (force) produced by each aorta ring of 2–3 mm obtained from the force transducers could be observed on an oscilloscope, digitalised, stored and analyzed as described previously (Dongmo et al., 2011). The following mouse lines were used for the experiments: wild type (WT) mice and mice lacking cGMP-dependent proteinkinase I selectively in smooth muscle (KO mice; Lukowski et al., 2008). These mice were provided by the Institute of Pharmacology and Toxicology of the Technical University of Munich and kept in the animal house of the institute. Animals weighing 20–25 g were used in the experiments. These animals were killed by decapitation in ether anaesthesia and the thoracic aorta was isolated and cleaned from connective tissue as described above. Aortic rings of 2–3 mm in width were mounted to organ baths (Myograph 601, Danish Myo Technology, Aarhus, Denmark. The bath contained modified Krebs–Henseleit solution. It was stirred continuously and aerated with 95% O_2 and 5% CO_2. The temperature was maintained thermostatically at 35 °C.

The resting force of the samples after mounting on the hooks was first set to 9.8 mN with a micromanipulator. The muscles were then equilibrated for 1 h with continuous changing of the bath solution until a constant base force was established, which was somewhat lower than the set resting force. To check the functional integrity of the preparations, contractions of the aortic rings were elicited by adding 30 mM KC1. Afterwards, the bath solution was changed until the resting tone was recovered (Vierling et al., 2003).

*Effect of the isolated **compound 2** (tetra-acetylajugasterone C, TAAC) on isolated rat aortic rings*

These experiments have been done only with **compound 2** (TAAC), since preliminary experiments showed that **compound 1** did not exhibit any vasorelaxant activity.

At first, the effect of **compound 2** which was identified as tetra-acetylajugasterone C, (TAAC) on noradrenaline- (NA-) or potassium-induced contraction of aortic rings was investigated. When the contractile response became stable after addition of the vasoconstrictor (1 μM NA or 60 mM KCl), the test substances were added in a cumulative manner to the organ bath solution. The relaxant effect of TAAC was determined by comparing the muscular tone of the contraction before and after addition of the test material. To determine the involvement of the NO/cGMP/cGKI pathway in the effect of TAAC, in some experiments, L-NAME (100 μM) or ODQ (10 μM) were added to the organ bath 15 min before elicitation of contraction of rat aortic rings with NA (1 μM) followed by the cumulative addition of TAAC reaching final bath concentrations of 0.01–100 μM. Additionally, for characterization of participation of the NO/cGMP/cGKI pathway, experiments were carried out using aortic preparations of mice with intact cGMP dependent protein kinase I (wild-type $cGKI^{+/+}$ mice) and of mice with knocked out cGKI ($cGKI^{-/-}$ mice). To evaluate the involvement of potassium (K^+) channels, tetraethylammonium (TEA; a non-selective potassium channel blocker, 10 mM), barium chloride (a K_{IR} blocker, 100 μM) or glibenclamide (a K_{ATP} blocker, 10 μM) were applied to rat vessel rings, 10 min prior to the contraction by NA (1 μM) followed by addition of TAAC (100 μM) during the resulting tonic contraction. The responses were compared with those obtained in vessels that had not been treated with these inhibitors. To determine an influence of TAAC on calcium influx of smooth muscle cells, arterial rings of rats were equilibrated in Ca^{2+}-free Krebs–Henseleit solution containing 0.2 mM EGTA and washed three times with this solution at 20 min intervals. NA (1 μM) was then added as a primer followed by cumulative addition of calcium (1–10 mM) at 10 min intervals. When maximum vasoconstriction was achieved, rings were again washed three times at 20 min intervals with Ca^{2+}-free solution. Then the arteries were incubated for 10 min with 100 μM TAAC or 0.1 μM nifedipine and thereafter again NA and calcium were added.

To differentiate between an influence of TAAC on calcium influx and intracellular calcium release, the effect on the two phases of noradrenaline-induced contraction was investigated. Rat arteries were contracted with 1 μM NA in the absence and the presence of 100 μM TAAC or 10 μM ryanodine in Ca^{2+}-containing Krebs–Henseleit solution. The phasic tension was measured as the peak contraction generated by noradrenaline, 10–15 s after agonist application; the tonic response was measured