Department of Animal Biology and Physiology[1] and Department of Organic Chemistry[2], Faculty of Science, University of Yaounde I, Yaounde, Cameroon, and Laboratoire de Pharmacologie et Physiopathologie Expérimentale[3], Université Montpellier I, Montpellier, France

Methanol extract of *Terminalia superba* induces endothelium-independent relaxation of rat thoracic aorta

T. Dimo[1], F. Laurent[3], S. V. Rakotonirina[1], P. V. Tan[1], P. Kamtchouing[1], E. Dongo[2], G. Cros[3]

Received February 23, 2005, accepted August 25, 2005

Prof. Dr. Theophile Dimo, Department of Animal Biology and Physiology, Faculty of Science, University of Yaounde I, P.O. Box 812, Yaounde, Cameroon
tdimo@uycdc.uninet.cm

Pharmazie 61: 470–473 (2006)

Terminalia superba is highly regarded in some parts of Cameroon in traditional medical practice. We have studied the vasorelaxant effects of the stem bark methanol extract of *T. superba* on rat vascular smooth muscle. The results demonstrated that *T. superba* extract provoked a time-dependent relaxation of aortic rings precontracted with norepinephrine (10^{-6} M). The vasorelaxant effect of the plant extract was not affected by endothelium removal or by pretreatment with indomethacin or N^w-nitro-L-arginine methyl ester (L-NAME). *T. superba* extract did not significantly, affect the contraction induced by 30 mM or 60 mM KCl as compared to those induced by NE. Relaxations elicited by *T. superba* extract were markely reduced by glibenclamide, a putative blocker for K_{ATP} channels and by tetraethylammonium, the non-specific K^+ channel inhibitor. *T. superba* caused a time- and concentration-dependent relaxation of the rat aortic rings that were inhibited by charybdotoxin and iberotoxin but not by apamin. These finding indicate that *T. superba* extract at least partially relaxes the rat aorta by activating K^+ channels, mainly K_{ATP} channels and large-conductance Ca^{2+}-activated K^+ channels in rat aorta.

1. Introduction

More than half of the species in the genus *Terminalia* appears throughout the dense humid forest, semi-decidious forest and easily flooded and secondary forest zones. In the south of Cameroon, numerous *Terminalia* species are employed in folk medicine for the treatment of gastroenteritis, diabetes, female infertility, abdominal pain and bronchial infections (Berhaut 1974; Adjanohoun et al. 1996). They have also been reported to possess antiplasmodial (Mustofa et al. 2000; Adewunmi et al. 2001), antidiabetic (Sabu and Kuttan 2002), and cardioprotective activities (Gauthaman et al. 2001). Phytochemical investigations of various species of *Terminalia* have revealed the presence of many classes of chemical constituents such as saponins, glycosides, flavonoids and chalchones (Srivastava et al. 2000).

The folk medical practice of some parts of the Center Province of Cameroon considers *T. superba* to be a useful remedy against hypertension. However, the literature is lacking in work that reports the cardiovascular activity of this species. The aim of the present study was to investigate the vasorelaxant activity of the dried methanolic extract of the stem bark extract of *T. superba* and to characterize its possible mode of action.

2. Investigations and results

Figures 1A and B show the relaxant effects of *T. superba* extract on rat aortic vascular preparations with and with-

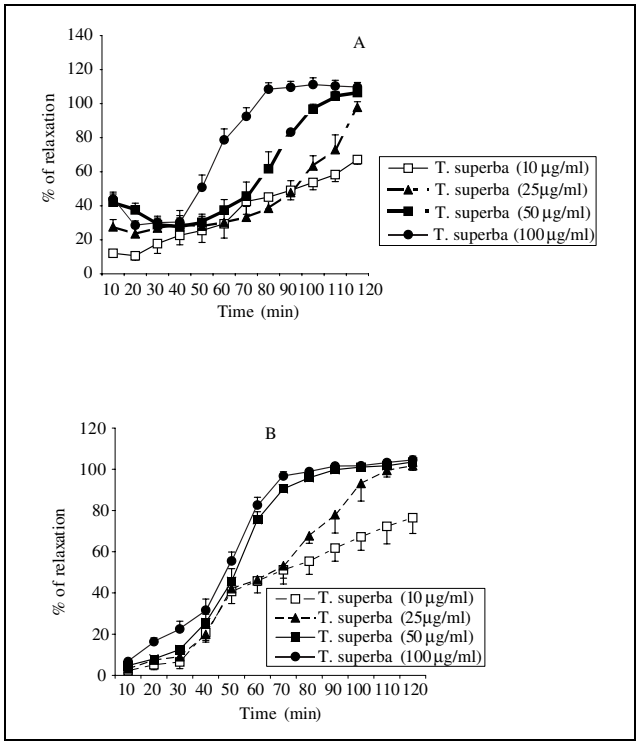

Fig. 1: Relaxant effects of *T. superba* extract on endothelium-intact (A) and-denuded (B) rat aortas precontracted by norepinephrine. Data are expressed as mean ± SEM, n = 5

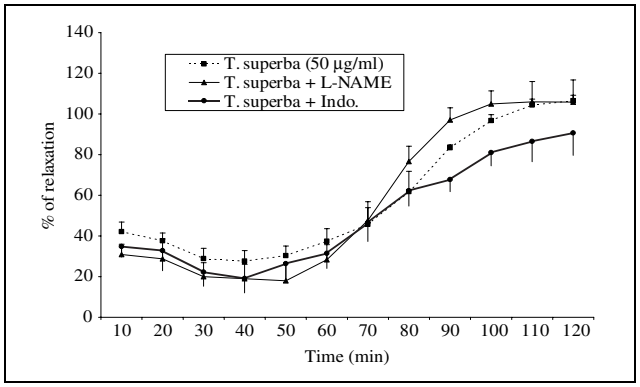

Fig. 2: Effects of *T. superba* on the relaxation kinetics of aortic rings treated with L-NAME or indomethacin (10^{-4} M). Data are expressed as mean ± SEM, n = 5. None of these agents significantly changed the time course of *T. superba*-induced relaxation

out endothelium precontracted with norepinephrin (NE, 10^{-6} M). The vasorelaxant effect of the methanol extract of *T. superba* was time-dependent. Concentrations ranging between 10–100 µg/ml yielded slowly evolving relaxations which achieved maxima over a period of 80 to 120 min depending on the extract concentration. The time taken to attain a 50% relaxation (t_{50}) was concentration-dependent. On the intact aortic rings, the t_{50} was 112 ± 6 min at the lower concentration of 10 µg/ml and was reduced to 53 ± 4 min at the higher concentration of 100 µg/ml. Removal of endothelium caused a significant reduction of t_{50} values (75 ± 6 min at 10 µg/ml and 45 ± 3 min at 100 µg/ml). There were no statistically significant differences between the times taken for 100% (t_{100}) relaxation to be attained on intact and denuded aortas precontracted with NE.

To assess the effect of L-NAME and indomethacin on endothelial function, the intact aortic rings were precontracted with NE and relaxation responses to a single concentration of *T. superba* (50 µg/ml) in the presence of N^w-nitro-L-arginine methyl ester (L-NAME) or indomethacin were determined. As shown in Fig. 2, the vasorelaxant kinetics of *T. superba* were not influenced by L-NAME or indomethacin (10^{-4} M).

The methanolic extract of *T. superba* did not significantly affect the contraction induced by 30 mM or 60 mM KCl. Relaxation due to 100 µg/ml of *T. superba* extract was 9.93 ± 1.73% and 6.29 ± 0.93% in rings precontracted with KCl (30 mM and 60 mM, respectively) as opposed to 109.82 ± 2.66% in those contracted with NE. The obvious dependence of the effects of *T. superba* extract on the transmembrane K^+ gradient suggests that K^+ channel currents is involved. For this reason, the effects of the methanol extract of *T. superba* in rat aortic rings, precontracted by NE, were studied in the presence of K^+ channel inhibitors and the results are shown in Fig. 3. In the presence of glibenclamide (10^{-6} M) or iberiotoxin (200 nM), the relaxation kinetics of *T. superba* were shifted to the right and the potency of the plant extract was as low as under control conditions. The maximal relaxation induced by 50 µg/ml of *T. superba* extract was reduced to 78.99 ± 2.70% and 97.73 ± 5.11%, respectively, compared to 106.54 ± 2.70% under control conditions. Tetraethylammonium (10^{-6} M) significantly reduced the relaxation in response to the methanol extract of *T. superba* during the first 40 min, but this inhibitory response was reversible as shown in Fig. 3. In the presence of apamin (500 nM), the effects of the plant extract remained unchanged while incubation with charybdotoxin (100 nM) had a significant

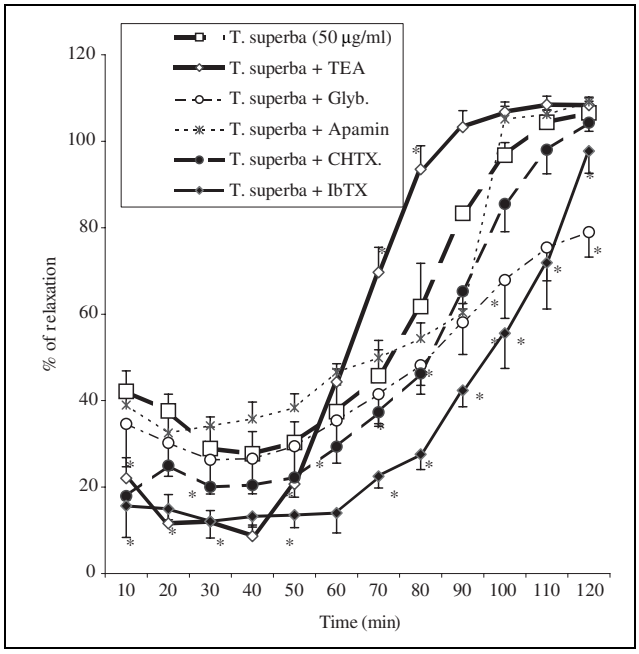

Fig. 3: Effects of tetraethylammonium (10^{-6} M), glibenclamide (10^{-6} M), apamin (500 nM), charybdotoxin (100 nM) and iberiotoxin (200 nM) on *T. superba*-induced relaxation of the isolated rat thoracic aorta. The values are given as mean ± SEM, n = 5, * $p < 0.05$ compared with the control vasorelaxation with 50 µg/ml *T. superba* extract

inhibitory effect on the relaxation induced by the extract during the first 10 min. The relaxation decreased from 42.14 ± 4.76% to 17.93 ± 0.90% in the presence of charybdotoxin.

3. Discussion

This study demonstrated that the methanol extract of *T. superba* induced a time-dependent relaxation, which was gradual, and reached a complete relaxation of aortic strips precontracted with NE after approximatively 70 to 120 min. The vasorelaxant effect of *T. superba* on NE-induced contraction was not affected by a prostaglandin synthesis inhibitor (indomethacin) or a nitric oxide synthase inhibitor (L-NAME) in intact rat thoracic aorta rings, which suggested that the effect was not mediated through either endothelium-derived prostacyclin or nitric oxide release from endothelial cells (Duarte et al. 1995; Campos et al. 2000). Endothelium removal did not affect, significantly, the relaxation induced by *T. superba* extract at t = 120 min, eliminating the possibility of the participation of any substance released by the endothelium. This suggests a direct effect of the plant extract on vascular smooth muscle and that the vasorelaxant effects of *T. superba* extract are independent of endothelium. Similarly, atriopepten III causes concentration dependent relaxation of all precontracted preparations with intact and with functionally destroyed endothelium indicating a direct effect on the smooth muscle cells. The relaxation is accompanied by an increase of cGMP (Vogel and Vogel 1997).

The first evidence of the involvement of K^+ channel-mediated hyperpolarization in the action of *T. superba* was provided by the differential potency of the extract in relaxing agonist-induced contractions instead of KCl-induced contractions. It is well known that vasodilators that depend on the K^+ channel mechanism lose their effects when exposed to high K^+ solutions because an increase in extracellular K^+ attenuates the K^+ gradient accross the

plasma membrane, thus rendering the K^+ channel-activating mechanism ineffective (Khan et al. 1997; Jackson 2000; Ceron et al. 2001). Our results confirm this because, when aortic strips were precontracted by high extracellular K^+, relaxations by *T. superba* extract were strongly reduced. This is supported by earlier studies which reported that nitroglycerin (Khan et al. 1998) and KMUP 880723, an aldehyde type α/β-adrenoceptor blocking agent (Chiu et al. 2000), produced vasorelaxation by K^+ channel activation-mediated hyperpolarization. Consistent with such a concept, K^+ channel openers such as cromakalim, pinacidil and nicorandil increase the permeability of the vascular smooth muscle cell membrane to K^+, resulting in a hyperpolarization that relaxes the blood vessel indirectly by decreasing the opening of voltage-sensitive Ca^{2+} channels (Kim et al. 1999). Thus, the decrease of the vasorelaxant effect of the plant extract in depolarized aortae, suggested that its inhibitory affect on Ca^{2+} entry diminishes when the inactivated state predominates. The failure of *T. superba* extract to relax KCl-precontracted rings may be due to the elimination of the chemical gradient for K^+ efflux, not impaired cGMP synthesis.

The present results show that *T. superba*-induced relaxation was significantly reduced by glibenclamide, a putative blocker for K_{ATP} channels (Yokoshiki et al. 1998; Lee et al. 2000), suggesting that ATP-sensitive K^+ channels in vascular smooth muscle contribute, at least in part, to the increased vasorelaxant effect of *T. superba*. It has been reported that the concentration of glibenclamide (10^{-6} M) used in this study was able to inhibit the vascular relaxations induced by compounds which possess the opening activity of K_{ATP} channels in a variety of vascular smooth muscles, including rat aorta (Saito et al. 1998). Tetraethylammonium, the non specific K^+ channel inhibitor (Huang 1998; Huang et al. 2001), attenuated during the first 40 min the relaxation response to the plant extract. Our results also demonstrated that apamin, a low-conductance Ca^{2+}-activated K^+ channel blocker (Kinoshita et al. 2000; Ceron et al. 2001) did not signifcantly influence the vasorelaxant kinetics of *T. superba*. Charybdotoxin attenuated the relaxant activity of the extract during the first 10 min while in the presence of iberiotoxin, another selective Bk_{ca} channel inhibitor (Archer et al. 1994; Khan et al. 1998; Jackson and Blair 1998; Seitz et al. 1999), the relaxation kinetics of *T. superba* extract was shifted to the right. It is therefore suggested that the methanol extract of *T. superba* at least partially relaxes the rat aorta by activating K^+ channels, mainly K_{ATP} channels and large-conductance Ca^{2+}-activated K^+ channels.

In conclusion, these results show that *T. superba* extract is able to produce vascular relaxation of the isolated rat aorta precontracted by NE probably due to activation of K^+ channels, and that this effect is endothelium-independent and time-dependent. The fact that K^+ channel inhibitors did not fully abolish the *T. superba*-induced vascular relaxation suggests that the activation of K^+ channels is not the only mechanism responsible for this activity. Other mechanisms may include activation of cyclic AMP elevating agents involved in vascular relaxation or stimulation of sarcolemmal and sarcoplasmic Ca^{2+} ATPase leading to a decrease in $[Ca^{2+}]_i$.

4. Experimental

4.1. Plant material

The stem bark of *T. superba* was harvested in the Ngoa Ekele II neighbourhood of Yaounde, Center Province, Cameroon, and authenticated at the national herbarium, Yaounde by comparison with herbarium voucher specimen N° 19 652/HNC earlier collected by Leeuwenberg. The sample was sun dried and the completely dried stem bark was powdered and used for extraction.

The dried powdered material (245 g) was macerated in a 1 : 1 (v/v) mixture of methylene chloride/methanol (CH_2Cl_2/CH_3OH, 2 L) for 2 days at room temperature. The resulting liquid extract was filtered through Whatman filter paper N°2 and concentrated under reduced pressure (bath temperature 80 °C). The solid obtained was sequentially washed with CH_2Cl_2 to give 42 g of the final methanol extract. This extract (2 g) was dissolved in 50 ml of physiological salt solution (PSS) to give a final concentration of 40 mg/ml. Dilutions of this solution in PSS were used in the experiments described below.

4.2. Tissue preparation and experimental protocol

Male Wistar rats (250–350 g) were killed by decapitation and their thoracic aortas were removed and placed in Krebs buffer solution containing, in mM; NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1. The adherent connective tissues and fat were cleaned and the aortas were cut into rings approximately 3–4 mm wide, care being taken not to damage the endothelium. In some experiments, the vascular endothelium was denuded intentionally by gently rubbing the internal surface using cotton thread. The aortic rings were suspended horizontally between two stainless steel wire hooks in organ chambers filled with 5 ml of physiological salt solution (PSS) (37 °C, pH 7.4) and bubbled with 95% O_2 and 5% CO_2. One wire was anchored to the plastic holder of the organ chamber and the other was connected to a transducer couple Narco Biosystem for the recording of the isometric tension. The aortic preparations were submitted to a basal tension of 1 g and were allowed to equilibrate for 60 min, during which the bath solution was renewed every 15 min. Functional integrity of endothelium was assessed by evaluating the ability of acetylcholine (ACh, 10^{-5} M) to produce relaxations of preparations precontracted with norepinephrine (NE, 10^{-8} M). Preparations were considered to contain a viable endothelium when ACh evoked relaxations exceeding 84% of precontraction, and were considered to be endothelium free when ACh. failed to cause a relaxation (Furchgott and Zawadski 1980; Cadene et al. 1997). After ACh testing, the aortic rings were washed with PSS three times during the next hour, prior to experimentation with the plant extract.

Following the equilibration period, the relaxation kinetics of aortic rings with and without endothelium were studied by contracting them with NE (10^{-6} M) for 30 min and then allowing them to relax in the presence of a single concentration of *T. superba* extract. The times taken for 50% (t_{50}) and 100% (t_{100}) relaxation to occur were recorded and used as indicators of the vascular relaxation potential of the extract.

To determine whether K^+ channels were involved in the relaxation induced by *T. superba* extract, the contractions were produced using a single concentration of 30 or 60 mM KCl in Krebs' solution. At the plateau of the contractile response induced by KCl, cumulative concentrations of the plant extract (from 10 to 100 μg/ml) were added. Experiments were also carried out in the presence of 100 nM charybdotoxin, 200 nM iberiotoxin, 500 nM apamin, 10^{-6} M tetraethylammonium and glibenclamide in order to study the inhibitory effects of these K^+ channel blockers.

Tissues were pretreated with K^+ channel blockers 45 min before contractions by NE, and then relaxations due to *T. superba* (50 μg/ml) were studied. In some experiments, a nitric oxide synthase inhibitor, N^w-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) or a prostaglandin synthesis inhibitor, indomethacin (10^{-4} M) were added 30 min before the addition of NE. Since the extract was evaporated to dryness and re-dissolved in PSS, it was not necessary to test for the residual effect of methanol.

4.3. Drugs

NE, acetylcholine, charybdotoxin, iberiotoxin, apamin, indomethacin, L-NAME, glibenclamide and tetraethylammonium were purchased from sigma Chemical Company (St. Louis, MO, USA). Ascorbic acid (0.57 mM) was added to each solution of NE, made up freshly every day. Glibenclamide was initially dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution and further dilutions made in PSS. The final amount of DMSO in the test solutions did not exceed 0.7% (v/v) and did not significantly affect the responses.

4.4. Expression of results and statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) and n represents the number of rats used in each experiment. Changes in aortic tension were expressed as a percentage relaxation of either NE-induced or KCl-induced tension. The one way analysis of variance (ANOVA) was used to determine the statistical significance of differences between treatments; $p < 0.05$ was considered to be statistically significant.

Acknowledgements: The authors wish to thank the Third World Academy of Sciences (TWAS) for the research grant N° 03-460/RG/BIO/AF/AC awarded to T. Dimo.

References

Adewunmi CO, Agbedahunsi JM, Adebajo AC, Aladesanmi AJ, Murphy N, Wando J (2001) Ethno-veterinary medicine: screening of Nigerian medicinal plants for trypanocidal properties. J Ethnopharmacol 77: 19–24.

Adjanohoun JE, Aboubakar N, Dramane K, Ebot ME, Ekpere JA, Enow-Orock EG, Focho D, Gbile ZO, Kamanyi A, Kamsu Kom J, Keita A, Mbenkum T, Mbi CN, Mbiele AL, Mbome IL, Mubiru NK, Nancy WL, Nkongmeneck B, Satabu B, Sofowora A, Tamze V, Wirmum CK (1996) Traditional medicine and pharmacopoeia. Contribution to ethnobotanical and floristic studies in Cameroon. Centre National de Production de Manuels Scolaires, Porto-Novo (Rep. Du Benin), p. 133.

Archer SL, Huang JMC, Hampl V, Nelson DP, Shultz PJ, Weir EK (1994) Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. Proc Natl Acad Sci 91: 7583–7587.

Cadene A, Grigorescu F, Serrano JJ, Cros G (1997) Characterization of vanadyl sulfate effect on vascular contraction: Role of calcium and tyrosine phosphorylation. J. Pharmacol Exp Ther 281: 491–498.

Campos MG, Oropeza MV, Villanueva T, Aguilar MI, Delgado G, Ponce HA (2000) Xanthorrhizol induces endothelium-independent relaxation of rat thoracic aorta. Life Sci 67: 327–333.

Ceron PIB, Cremonez DC, Bendhack LM, Tedesco AC (2001) The relaxation induced by S-nitroso-glutathione and S-nitroso-N-acetylcysteine in rat aorta is not related to nitric oxide production. J Pharmacol Exp Ther 298: 686–694.

Duarte J, Pérez-Vizcaíno F, Torres AI, Zarzuelo A, Jiménez J, Tamargo J (1995) Vasodilator effects of visnagin in isolated rat vascular smooth muscle. Eur J Pharmacol. 286: 115–122.

Furchgott RF, Zawadzki JV (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288: 373–376.

Gauthaman K, Maulik M, Kumari R, Manchanda SC, Dinda AK, Maulik SK (2001) Effect of chronic treatment with bark of Terminalia arjuna: a study on the isolated ischemic-reperfused rat heart. J Ethnopharmacol 75: 197–201.

Huang Y (1998) Hydroxylamine-induced relaxation is inhibited by K^+ channel blockers in rat aortic rings. Eur J Pharmacol 349: 53–60.

Huang Y, Bourreau JP, Chan HY, Lau CW, Wong JWT, Yao X (2001) Inhibitory effect of tetrabutylammonium ions on endothelium/nitric oxide-mediated vasorelaxation. Life Sci 69: 1661–1672.

Jackson WF (2000) Ion channels and vascular tone. Hypertension 35 (part 2), 173–178.

Jackson FW, Blair LK (1998) Characterization and function of Ca^{2+}-activated K^+ channels in arteriolar muscle cells. Heart Cir Physiol 274: H27–H34.

Khan SA, Higdon NR, Meisheri KD (1998) Coronary vasorelaxation by nitroglycerin: involvement of plasmalemmal calcium-activated K^+ channels and intracellular Ca^{++} stores. J Pharmacol Exp Ther 284: 838–846.

Kim DN, Kang YS, Kim MJ, Park HJ, Schini-Kerth BV (1999) The ginseniside Rg3 evokes endothelium-independent relaxation in rat aortic rings: role of K^+ channels. Eur J Pharmacol 367: 51–57.

Kinoshita H, Ishikawa T, Hatano Y (2000) Rôle of K^+ channels in augmented relaxations to sodium nitroprusside induced by mexiletine in rat aortas. Anesthesiology 92: 813–820.

Lee YM, Yen MH, Peng YY, Sheu JR, Chen YC, Chang MJ, Cheng CY (2000) The antihypertensive and cardioprotective effects of (−)-MJ-451, an ATP-sensitive K^+ channel opener. Eur J Pharmacol 397: 151–160.

Mustofa AV, Benoit-Vical F, Pélissier Y, Koné-Bamba D, Mallié M (2000) Antiplasmodial activity of plant extracts used in west african traditional medicine. J Ethnopharmacol 73: 145–151.

Sabu MC, Kuttan R (2002) Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property. J Ethnopharmacol 81: 155–160.

Saito W, Aida M, Sasaki M, Saito Y, Tanaka Y, Tanaka H, Shigenobu K (1998) Differential sensitivity to ATP-sensitive potassium channel openers of norepinephrine-induced contraction of guinea pig and rat aorta. Life Sci 62: 2171–2179.

Seitz S, Wegener JW, Rupp J, Watanabe M, Jost A, Gerhard R, Shainberg A, Ochi R, Nawrath H (1999) Involvement of K^+ channels in the relaxant effects of YC-1 in vascular smooth muscle. Eur J Pharmacol 382: 11–18.

Srivastava SK, Srivastava SD, Chouksey BK (2001) New antifungal constituents from *Terminalia alata*. Fitoterapia 72: 106–112.

Tanaka Y, Igarashi T, Kaneko H, Yamaki F, Mochizuki Y, Aida M, Taniguchi H, Tanaka H, Shigenobu K (2000) NO-mediated maxi K_{Ca} channel activation produces relaxation of guinea pig aorta independently of voltage-dependent L-type Ca^{2+} channels. Gen Pharmacol 34: 159–165.

Yokoshiki H, Sunagawa M, Seki T, Sperelakis N (1998) ATP-sensitive K^+ channels in pancreatic, cardiac, and vascular smooth muscle cells. Am J Physiol 274: C25–C37.

Vogel HG, Vogel HW (1997) Drug discovery and evaluation. Pharmacological assays. Springer-Verlag Berlin Heidelberg, Germany, p. 41.