



***Bauhinia candicans* improves the endothelium-dependent relaxation in aortic rings of alloxan-diabetic Rats.**

[*Bauhinia candicans* mejora la relajación dependiente de endotelio en anillos de ratas diabético-aloxánicas]

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Abstract

Bauhinia candicans is a plant used in Chile for diabetes management. The aim of the present study was to investigate the relaxing responsiveness to acetylcholine (ACh) of phenylephrine-precontracted aortic rings from alloxan-diabetic rats treated with an aqueous-ethanol extract of *B.candicans* leaves (EBc-rats, 120 mg/kg/day). EBc significantly reduced fasting blood sugar from 292.4 ± 9.2 mg/dl to 174.6 ± 12.8 mg/dl. ACh induced a negligible reduction of tension ($28 \pm 6\%$) of aortic rings from ALX-rats compared with EBc-rats ($48 \pm 5.5\%$) and normal ($57 \pm 5\%$). The enhanced sensitivity to phenylephrine in aortic rings from diabetic rats was reduced in EBc-rats. Removal of the endothelium or pretreatment of rings of EBc-rats with nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) increased the Phe response. The nitric oxide (NO) donor sodium nitroprusside (SNP) induced a normal relaxation in these rings. The results suggest that the extract of leaves of *B. candicans* increases endothelium-dependent relaxation of aortic rings of ALX-rats and its use may be helpful in the prevention of diabetic complications.

Keywords: *Bauhinia candicans*; diabetes; endothelial dysfunction; nitric oxide

Resumen

Bauhinia candicans es una planta usada en Chile para el tratamiento de la diabetes mellitus. Se investigó el efecto de un extracto acuoso-etanólico de las hojas de *Bauhinia candicans* (EBc, 120 mg/kg/día) sobre la respuesta relajadora de acetilcolina en anillos aórticos de ratas diabético-aloxánicas precontractados con fenilefrina. EBc redujo la glicemia de ayuno de 292.4 ± 9.2 mg/dl a 174.6 ± 12.8 mg/dl. Acetilcolina indujo una pequeña relajación ($28 \pm 6\%$) de los anillos de ratas diabéticas comparadas con los de ratas tratadas con EBc (ratas-EBc; $48 \pm 5.5\%$) y con los de ratas normales ($57 \pm 5\%$). La alta sensibilidad a Phe en aorta de ratas diabéticas fue reducida en ratas-EBc. La remoción del endotelio y la incubación con L-NAME de los anillos de ratas-EBc generaron una alta respuesta a Phe. Nitroprusiato de sodio (SNP) indujo una relajación normal en estos anillos. Los resultados sugieren que el extracto de hojas de *B. candicans* aumenta la relajación dependiente de endotelio de los anillos de aorta de ratas diabéticas y su uso puede ser útil en la prevención de las complicaciones de la diabetes.

Palabras Clave: *Bauhinia candicans*; diabetes; disfunción endotelial; óxido nítrico

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INTRODUCTION

Diabetes is associated with atherosclerotic macrovascular disease affecting arteries that supply the heart, brain and lower extremities (Brownlee, 1991). A much higher risk of myocardial infarction, stroke, and limb amputation is related to endothelial dysfunction (ED) (Ostlund et al., 1993). Hyperglycemia and hyperlipidemia are risk factors for ED and can induce ED *in vitro* and *in vivo* (Tefamariam et al., 1991). Hyperglycemia-induced ED may result from decreased production of NO, inactivation of NO by oxygen-derived free radicals, and/or increased sensitivity to vasoconstrictors such as angiotensin II and endothelin (Kamata et al., 1992). Nitric oxide formed via endothelial NO synthase (eNOS) plays crucial roles in the regulation of blood flow through vasodilatation and decreased vascular resistance in various organs and tissues (Ignarro et al., 1999).

Oral hypoglycemic agents and insulin are widely used for lowering blood sugar in diabetes, but these drugs have various side effects and can bring liver and renal damage (Derosa and Maffioli, 2010). Oxidative stress is known to play a significant role in the induction of these processes (Novo and Parola, 2009). Ethnobotanical studies indicate that the decoctions of the leaves and stem bark from *Bauhinia candicans* (folk name: "pata de vaca"), a plant widely distributed in South American countries, are used to treat diabetes mellitus (Lemus et al., 1999). Chemical studies of *B. candicans* and other species of this genus, have been showed that these plants, are a rich source of flavonoids particularly flavonols glycoside (Jorge et al., 2004; Da Silva and Cechinel, 2002). However, no studies to the pharmacological properties regarding of these *B. candicans* compounds have been reported. In a previous work, using normal and experimentally induced diabetic rabbits, we reported the hypoglycemic effect of different fractions of the methanolic extract of *B. candicans* leaves (Fuentes et al., 2004). Moreover, other studies (Fuentes and Alarcón, 2006) using isolated gastric glands of normal and diabetic rabbits, have shown an increased glucose transport at the vasolateral level of the gastric cells in the presence of the butanol extract of *B. candicans*

The aim of this study was to check the hypothesis that the hypoglycaemic condition generated by *B. candicans* treatment reverts the functional impairment of the endothelium dysfunction and NO synthesis

generated by the hyperglycemia. To achieve this, aortal rings of alloxan-induced diabetic rats incubated in Krebs solution were tested in *in vitro* experiments.

MATERIALS AND METHODS

Plant material

B. candicans (Leguminosae) leaves were collected in December 2008 in Chillán (VIII Region, Chile). Botanical authentication was confirmed by Prof. Victor Finot of the Department of Animal Production, Faculty of Agronomy, University of Concepción, Chile. A voucher specimen has been deposited in the herbarium of Department of Basic Sciences, University of Bio-Bio, Chillan, Chile.

Extraction and preliminary screening

The aqueous ethanol extract was prepared as follows: 700 g of the chopped and dried leaves of *B. candicans* were extracted with 2L distilled water and 2L ethanol. The filtered and evaporated extract gave a dry residue (yield: 12.5% w/w). The residue was dissolved in saline solution to achieve a final concentration of 10 g/100 ml. Preliminary chemical study has allowed to isolate and identify four known flavonoids: kaempferol-3,7-*O*- α -L-dirhamnoside, kaempferol-3-*O*- β -D-glucopyranosyl-(6 \rightarrow 1)- β -L-rhamnopyranosyl-7-*O*- α -L-rhamnopyranoside, quercetin-3,7-*O*- α -L-dirhamnoside and quercetin-3-*O*- β -D-glucopyranosyl-(6 \rightarrow 1)- β -L-rhamnopyranosyl-7-*O*- α -L-rhamnopyranoside. The structure of the compounds was established on the basis of spectroscopy data, acidic hydrolysis and comparison with authentic sample.

Animals

The present study used Albino Wistar rats of either sex with weights in the range of 180-250 g. Females rats were used because changes in endocrine status did not affect vascular function in the arteries during different oestrus states (Santmyre et al., 2010; Uematsu et al., 2010). The animals were obtained from central animal house, Departamento de Ciencias Básicas, Universidad del Bío-Bío, Chillán, Chile, and were housed in an air-conditioned colony room at 23 \pm 4 °C with 12 h light and 12 h dark cycles. The control and experimental animals were provided food and water *ad libitum*. Standard pellets obtained from Purina Lab Chow were used as a basal diet during the experimental period. All the animals were carefully monitored and maintained in accordance with the ethical recommendation of the

National Institute of health Guide for Care and use of Laboratory Animals.

Bioassay

Diabetes mellitus was induced by a single intraperitoneal injection of freshly prepared solution of alloxan (ALX) monohydrate (60 mg/kg) in saline solution after overnight fasting. After five days, animals were considered diabetic if their blood glucose values were between 200 and 300 mg/dl. Then, animals were randomly allocated into three groups of 12 to 15 rats each. Group 1, 2-week ALX-treated rats, were orally dosed daily with *B. candicans* extract (EBc-rats, 120 mg/kg/day); group 2, 2-week ALX-treated rats (ALX-diabetic) and group 3, nondiabetic- control, received only saline. The groups received the respective treatment at 9:00 h during 14 days. Blood glucose levels were determined by the glucose dehydrogenase method using a glucometer (Accu-Chek Sensor Comfort, Roche Diagnostics, Mannheim, Germany)

Drugs

Phenylephrine, Acetylcholine chloride, alloxan monohydrate, N(G)-nitro-L-arginine methyl ester, sodium nitroprusside (SNP), and all the other reagents and compounds used for Krebs' solution were purchased from Sigma (St. Louis, MO, USA). Drugs were dissolved in distilled water to prepare stock.

Aortic rings

Animals of 2-week were anesthetized with diethyl ether and killed with a small animal guillotine. The thoracic aorta was immediately excised and placed in Krebs' solution of the following composition (mM): NaCl 118, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 25 and glucose 11.1. The thoracic aorta was carefully cleaned of adhering fat; connective tissue was removed and then cut into transverse rings (3–4 mm). Aortic rings were mounted, under 2 g resting tension, in an organ bath (Radnoti, Monrovia Ca, USA) containing 50 ml Krebs' solution and maintained at 37°C, pH 7.4, gassed with 95% O₂ and 5% CO₂. Isometric measurement were recorded with a transducer (Radnoti) and displayed on PowerLab software (AD Instruments, USA). The tissue was allowed to equilibrate for 60 min before carrying out the experiments, during which time the resting tension was readjusted to 2 g as required. The aortic rings were submaximally contracted with 1 μM Phe. The

functional presence of endothelium was verified by the ability of 1 μM Ach to induce relaxation, and endothelium-denuded preparation were used to confirm the involvement of NO with sodium nitroprusside (SNP, 10 nM).

Data analysis and statistical methods

The contractile responses of aortic rings were expressed as percentages of change respect to their own maximum tension (grams) to 1 μM phenylephrine. Vascular responses to the vasodilator Ach were reported as the percentage of reduction in tension (remaining contraction) compared with the tone level induced by contraction with Phe. The experimental results (usually 2–4 from each animal) were averaged and used in subsequent analyses. Results are expressed as the mean ± S.E.M. Student's *t*-test was used to analyze the significance of the results. Values of *p* < 0.05 were considered significant.

Results and Discussion

It is well established that *Diabetes mellitus* is associated with the development of vascular dysfunction. Previous reports have shown that acute or chronic hyperglycemia can brought vascular abnormalities, such as an impairment of endothelium-dependent vasodilatation and increase in the response to vasoconstrictor agents (Majithiya and Balaraman, 2006). The involvement of the vascular endothelium in the relaxation of arterial smooth muscle caused by NO release, which is stimulated by acetylcholine, has been well demonstrated (Ignarro et al., 1999).

In the present study, we examined the relationship between diabetes-induced hyperglycemia and endothelium-mediated arterial function in rats treated with EBc.

The results demonstrated that the extract of *B. candicans* leaves exerted a significant (*P* < 0.001, *t* test) anti-hyperglycaemic activity in ALX diabetic (Fig 1). The effect of EBc was first demonstrated in rabbits (Fuentes et al., 2004) and the mechanism by which this extract lowers blood glucose may be associated with the increased peripheral utilization of glucose (Fuentes and Alarcón, 2006).

In endothelium-intact preparations of ALX-rats (Fig.2), contractions elicited by phenylephrine were

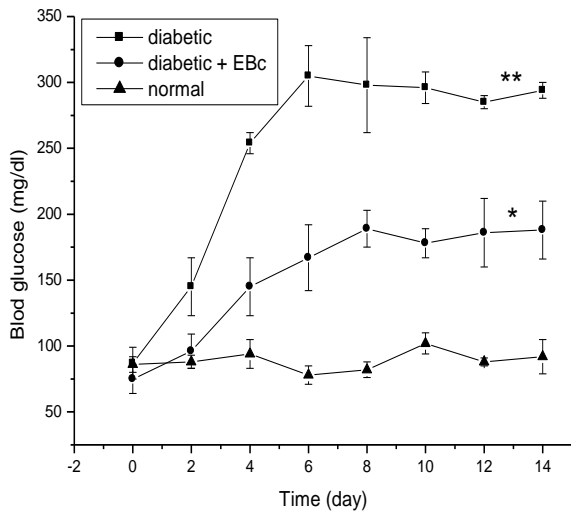


Figure 1. Blood glucose concentration in alloxan-diabetic rats treated orally with EBc (120 mg/kg/day). Glucose values are expressed as a mg/dl (n= 12). The values represent mean ± S.E.M. Significance: * $P < 0.005$ vs ALX-diabetic, ** $P < 0.001$ vs normal, Student’s *t*-test.

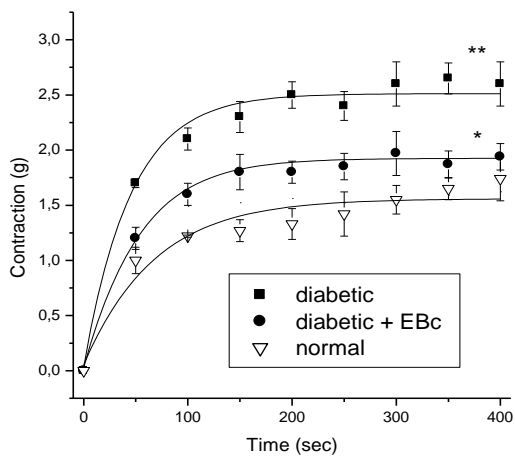


Figure 2. Time-course of phenylephrine (1 µM) contraction of aortic rings of normal, ALX-diabetic and ALX-diabetic + EBc rats. Each point indicate the mean ± S.E. (n= 12), Significance: $P^* < 0.005$ vs ALX-diabetic, $P^{**} < 0.001$ vs. normal, Student’s *t*-test. significantly enhanced (44 ± 8.5 %, n = 12) of the control responses. Furthermore, enhanced Phe-induced contraction in ALX-diabetic rats was reduced by EBc treatment ($P^* < 0.005$ vs ALX-diabetic). The mechanisms responsible for an increased contractile

response of diabetic arteries to vasoconstrictor agonists are not completely understood, although alterations of endothelial function, enhanced calcium mobilization and inhibition of Na^+ , K^+ -ATPase activity appear to contribute to these changes in vascular reactivity (Xavier et al., 2003). The increased responsiveness of diabetic vessels to exogenous phenylephrine also seems to be related to depressed basal NO bioavailability that could be considered as a compensatory way against activated contractile mechanisms of diabetic vascular smooth muscle. This presumption is supported by our observation that the additional endothelium-dependent increment of the Phe-induced contraction, produced in the presence of L-NAME, is significantly enhanced in 2-week EBc-treated diabetic rats (Fig. 4E). Our results reinforce similar findings showing a decreased production of NO in the diabetic state (Olukman et al., 2010).

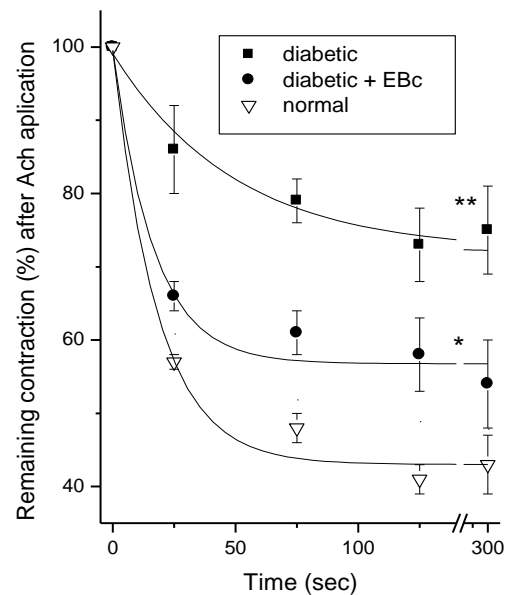


Figure 3. Effect of *Bauhinia candicans* (EBc) on the relaxation curves to acetylcholine in aorta. Contraction was evoked by phenylephrine (1 µM). Acetylcholine (1 µM) was added into the solution when the contraction had reached a plateau. Relaxation is presented as the percentage of the contraction remaining after addition of the agent to contracted rings isolated from normal, ALX-diabetic and ALX-diabetic+EBc rats. Each point is the mean from 7-10 determinations. Significance: $P^* < 0.005$ vs ALX-diabetic, $P^{**} < 0.001$ vs normal, Student’s *t*-test

After 2 weeks of diabetes, aortae of rats contracted submaximally by Phe (Fig.3), showed significantly decreased endothelium-dependent relaxations induced by Ach compared with the aortae of normal rats ($P^{**}<0.001$). The figure also show that Ach-induced relaxation of the aortic rings of alloxan-diabetic rats, treated with EBc, was greater than ($P^* <0.005$) the relaxation observed in ALX-diabetic untreated rats. As shown in fig. 4 (D to F) the use of exogenous NO (SNP) demonstrated that the altered vascular response to Ach in diabetic conditions may be due to an endothelial dysfunction rather than a muscle cell damage.

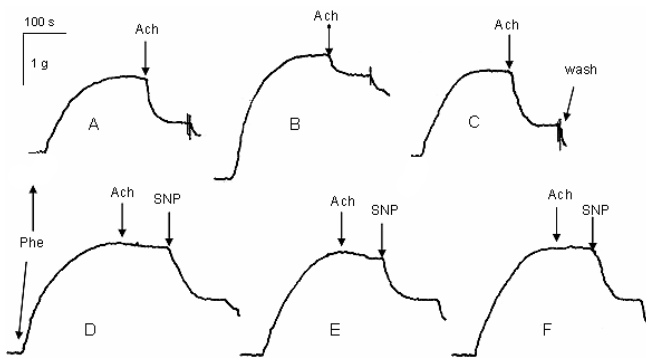


Figure 4. Original records of time-course changes in endothelium-dependent and -independent relaxation of aortas from: A = normal rat; B = diabetic rat; C = diabetic BCE-treated rat; D = endothelium-denuded diabetic rat; E = endothelium-intact plus L-NAME, BCE-treated diabetic rat. SNP = sodium nitroprusside and F = endothelium-denuded BCE diabetic rat considering that the relaxation induced by Ach in the rat aorta is mainly dependent on NO production, our data suggests that the enhanced Ach-dependent relaxation observed in ALX-diabetic rats treated with EBc was due to an increasing in the sensitivity to NO production rather than a direct relaxing effect of EBc on arterial smooth muscle. This presumption is partially based by others studies in our laboratory using both, Phe precontracted aortae from ALX-rats treated with L-NAME and aortae without endothelium, wich show no change in tension in the presence of EBc (results not shown).

The ability of antidiabetic plants to inhibit lipid peroxide formation and to scavenge hydroxyl and

superoxide radicals in vitro were observed in a recent study (Sabu and Kuttan, 2002), therefore, the effect of *Bauhinia forficata* (Syn.: *Bauhinia candicans*) could be explained by its antioxidant activity, which was recently described by Khalil el al. (2008), due to the fact that endothelium-derived nitric oxide rapidly reacts with O_2^- (rate constant 2×10^{10} L/mol per s) which reduces its relaxant activity (Kissner et al., 1997; Hogg, 1998).

Conclusion

in conclusion, the present study demonstrated that the ebc possess significant antihyperglycemic effect in rats at the doses investigated. the results also suggest, that daily ebc administrations ameliorate endothelial dysfunction in alx-diabetic rats, and that the nitric oxide pathway may mediate the effect of ebc. further studies are in fact currently under way to isolate and characterize the active principle (s) of the extract.

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