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Maqui (*Aristotelia chilensis*) and rutin (quercetin-3-O-rutinoside) protects against the functional impairment of the endothelium-dependent vasorelaxation caused by a reduction of nitric oxide availability in diabetes

[El Maqui (*Aristotelia chilensis*) y la rutina (quercetin-3-O-rutinoside) protegen contra el deterioro funcional de la vasorelajación dependiente de endotelio causada por la reducción de la disponibilidad de óxido nítrico en diabetes]

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Abstract
The present study evaluated the beneficial effect of hydroalcoholic extract of maqui berry (*Aristotelia chilensis*, Elaeocarpaceae) and rutin (quercetin-3-o-rutinoside) against vascular reactivity impairment, hyperglycemia and dyslipidemia of alloxan (alx) diabetic rats. The chronic maqui berry extract (mbe) treatment significantly corrected all the above abnormalities in diabetic rats. Rutin reduced fasting blood sugar and improved the impaired endothelium-dependent relaxations. Maqui reduced plasma levels of cholesterol, LDL and triglycerides and increased body weight of diabetic rats. Removal of the endothelium and nitric oxide synthase (NOS) inhibitor, NG-Nitro-L-Arginine Methyl Ester (L-NAME) increased the phenylephrine response, and sensitivity to sodium nitroprusside (SNP) did not differ between tested groups. Maqui and rutin improved nitric oxide bioavailability, and these findings indicate that *Aristotelia chilensis* could be a candidate of natural medicine for diabetes.

Keywords: *Aristotelia chilensis*, diabetes, endothelial dysfunction

Resumen
El presente estudio evaluó el efecto beneficioso del extracto hidroalcohólico de maqui (*Aristotelia chilensis*, Elaeocarpaceae) y rutina (quercetina-3-o-rutinósido) contra el deterioro de la reactividad vascular, hiperglucemia y dislipidemia de ratas diabéticas. El tratamiento crónico con el extracto corrigió en gran medida esas alteraciones. Rutina redujo el azúcar en sangre y mejoró la relajación dependiente de endotelio. Maqui redujo los niveles plasmáticos de colesterol, LDL y triglicéridos y aumentó del peso de las ratas diabéticas. La eliminación del endotelio y el inhibidor de la sintasa de óxido nítrico, NG-Nitro-L-Arginina Metil Éster (L-NAME) aumentaron la respuesta a la fenilefrina y, la sensibilidad al nitroprusiato de sodio, no cambió entre los diferentes grupos. Maqui y rutina mejoraron la biodisponibilidad del óxido nítrico. Estos hallazgos indican que *Aristotelia chilensis* podría ser un candidato de la medicina natural para la diabetes.

Palabras Clave: *Aristotelia chilensis*, diabetes, disfunción endotelial

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INTRODUCTION

Diabetes mellitus (DM) is a highly prevalent chronic metabolic disorder that is considered a major health problem in westernized societies (Deshpande *et al*., 2008). The prevalence of diabetes is increasing dramatically around the world, and in some areas has reached epidemic proportions. Diabetes, characterized by persistent elevation of blood glucose levels (hyperglycemia), occurs due to an inadequate production of insulin (type 1 diabetes), or resistance to endogenous insulin (type 2 diabetes). Despite intensive glycemic control, diabetic individuals are predisposed to developing different complications such as cardiomyopathy, retinopathy, atherosclerosis, neuropathy and nephropathy (Sharma *et al*., 2012). Besides, abnormal production of reactive oxygen species (ROS) in endothelial cells of large and small vessels has been implicated in various chronic diseases, including diabetes (Folli *et al*., 2011) and hypertension (Cahill-Smith and Li, 2011). Together with other vascular complications, including diminished production of nitric oxide (NO), the increased levels of ROS play a key role in the pathogenesis of micro- and macrovascular diabetic complications (Sharma *et al*., 2012). NO, produced by vascular endothelial cells, is recognized as a relaxing, anti-inflammatory and anti-atherogenic principle in the vasculature (Tousoulis *et al*., 2012). Decreased availability of NO in the vasculature promotes the progression of cardiovascular diseases in diabetic people. The term "endothelial dysfunction" (ED) in fact, refers to impairment of endothelium-dependent vasorelaxation caused by a reduction of nitric oxide availability due to an accelerated degradation by ROS (Hua *et al*, 2008).

There is growing evidence that several single natural compounds and plant extracts modulate endothelial NO production (Stoclet and Schini-Kerth, 2011). Different antioxidants and/or scavengers of oxygen free radicals were shown to activate nitric oxide synthase (NOs) and to increase the expression of both endothelial and neuronal NOs isoforms leading to blood pressure reduction (Kojsová *et al*., 2006). Therefore, identification of those active compounds and descriptions of their cellular actions may improve our knowledge of the regulation of endothelial NO production and can provide important insight for the prevention or treatment of cardiovascular alterations generated in diabetes. Animal and clinical studies have suggested that polyphenols from plants may delay the development of endothelial dysfunction through their antioxidant and anti-inflammatory properties (Schini-Kerth *et al*., 2011).

Maqui (*Aristotelia chilensis* (Molina) Stunz, Elaeocarpaceae), is a plant with purple colored berry natural from Patagonia region of South America. Native Indians (Mapuche ethnia) consume maqui berries (MB) as food, as medicine and have done so for centuries. Some studies have shown that Mapuche children have less chance of developing insulin-dependent *diabetes mellitus* than caucasian children (Larenas *et al.,* 1996) and the prevalence of type 2 diabetes and dyslipidemia in urban aboriginal populations is higher than that of their rural patagonian counterparts (Carrasco *et al.,* 2006). Besides being frequently consumed for their delicious and unique flavor, they use this berry to treat health complaints such as skin inflammation, intestinal disorders, sore throats, wound healing and ulcers (Mølgaard *et al*., 2011). But, there is not folk knowledgement of the beneficial properties of continuous use of maqui as a potentially protective source against chronic diseases such as diabetes and hypertension and cancer (Schreckinger *et al*., 2010). Recent studies have shown that Maqui berry has one of the highest Oxygen Radical Absorbance Capacity (ORAC) values compared to any other fruits and berries (ORAC-H value of 27600 μmole, which is double than Acai berries and threefold than pomegranate (Rubilar *et al*., 2011). From water and ethanol extract and their fractions of mature fruits of MB, different phenolic compounds were identified by chromatographic (HPLC) and unequivocally assignments by spectroscopic (UV, NMR) data analysis (Céspedes *et al*., 2010). Other studies on MB revealed that this fruit has a high concentration of polyphenols and anthocyanins (Céspedes *et al*., 2009; Schreckinger *et al*., 2010, Rubilar *et al*., 2011, Escribano-Bailón *et al*., 2006)), which may be the reason for the high antioxidant activity observed in MB (Ruiz *et al*.,2011). Many of the compounds present in MB, have shown great potential health benefits (Céspedes *et al*., 2010; de Haan and Cooper, 2011) however, it is necessary to know more about their properties to propose suitable applications. The aim of the present study was to investigate whether an hydroalcoholic extract of maqui berry (MBE) and the polyphenolic flavonoid quercetin-3-O-rutinoside (Rutin), synthesized in the plant, protects against the functional impairment of the endothelium-dependent vasorelaxation caused by a reduction of NO availability in diabetes. Rutin, a flavonoid commonly found in higher plants, has

numerous health benefits including neuroprotective effects (Nakayama *et al*., 2011), attenuated renal inflammation and apoptosis (Arjumand *et al*., 2011), reduced risk of cancer (Han *et al*., 2009) , reduced incidence of coronary heart disease (Fernandes *et al*., 2010) and increased life expectancy. Rutin by its ability to scavenge free radicals and to inhibit lipid peroxidation, prevents streptozotocin-induced oxidative stress and protects β-cells resulting in increased insulin secretion and decreased blood glucose levels (Kamalakkannan and Prince, 2006a).

MATERIAL AND METHODS

Chemical and solvent

All enzymatic reagents were from DiaSys Diagnostic Systems GmbH (Germany). Phenylephrine, Acetylcholine chloride, alloxan monohydrate, N(G)-nitro-L-arginine methyl ester, sodium nitroprusside (SNP), dimethyl sulfoxide (DMSO) and all the other reagents and compounds used for Krebs' solution were purchased from Sigma (St. Louis, MO, USA).

Plant material

The fruits of *Aristotelia chilensis*, MB, matured in summer, were collected from Rinconada, a golf court besides Cato River in the central valley of *Provincia de Ñuble, Biobío Region*, Chile, and identified by Dr. 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 the Department of Basic Sciences of University of Bio-Bio, Chillán, Chile.

The aqueous ethanol extract was prepared by adding EtOH-H_2O (6:4) to 430 g of dried and ground pulp of MB. After maceration, the extract was filtered on Whatman paper, and the solvent was evaporated under reduced pressure (50 °C) in a rotary evaporator. Thus, the crude hydro-alcoholic extract resulted in a dark purple residue (MBE, yield 32 g) stored at RT until use.

Phytochemical screening of MBE revealed the presence of gentisic acid, ferulic acid, gallic acid, p-coumaric acid, sinapic acid, 4-hydroxybenzoic acid, delphinidin, cyanidin, vanillic acid, delphinidin gallate, gallocatechin gallate, quercetin, rutin, myricetin, catechin and epi-catechin as mixture 1:1, and several glycosides of anthocyanidins (delphinidin-3-sambubioside-5-glucoside, delphinidin-3,5-diglucoside, cyanidin-3-sambubioside-5-glucoside, cyanidin-3,5-diglucoside, delphinidin-3-sambubioside, delphinidin-3-glucoside, cyanidin-3-sambubioside, and cyanidin-3-glucoside), and proanthocyanidin B. as reported by Céspedes *et al*. (2010).

Biochemical determinations

Serum glucose was measured according to the glucose dehydrogenase method using a glucometer (Accu-Chek Sensor Comfort, Roche Diagnostics, Manheim, Germany). Total cholesterol, LDL- cholesterol and HDL-cholesterol levels were determined by homogeneous enzymatic colorimetric test using Hitachi Auto analyzer 917/MODULAR P provided by Boehringer Mannheim/Hitachi, (Germany/Japan) as previously described (Steiner *et al*., 1981). Plasma was mixed with corresponding auto reagents and absorbance was measured at 500 ηm. Triglycerides were determined by colorimetric enzymatic test using glycerol-3-phosphate oxidase.

Experimental Animals

Wistar male rats (8-11 months) weighing in the range of 180-250 g were used for the study. The animals were obtained from central animal house, *Departmento de Ciencias Básicas, Universidad del Bio-Bío*, Chillán, Chile, and were housed in an air conditioned colony room at 23 ± 4 °C with 12h light and 12h dark cycles. The control and experimental animals were given food and water ad libitum. Standard pellets obtained from Purina Lab Chow were used as a basal diet during the experimental period. The study was performed under the supervision of the Ethical Committee of the Biobío University in Chillán, Chile and in conformity with "The Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

Induction of type 2 diabetic rat model and treatment

Diabetes mellitus was induced in rats, by a single intraperitoneal injection of freshly prepared solution of alloxan (ALX) monohydrate (80 mg/kg) in a saline solution after overnight fasting. After five days the animals were considered diabetic if their blood glucose values were over 200 mg/dl. Then the animals were randomly allocated into four groups of 10 rats each. Group I, 3-week nondiabetic-control (normal), received only saline by oral gavage; group II, 3-week ALX-diabetic rats, received MBE at dose level of 50 mg (dissolved in saline with dimethyl sulfoxide, DMSO, 5%)/Kg b.w. by oral gavage (DMBE rats) twice daily. Group III, diabetic-control rats (DC), were administered saline with the equivalent volume of the vehicle (DMSO) for the same period by the same

route; and group IV, 3-week ALX-treated rats, were orally dosed with normal saline with 30 mg/kg rutin dissolved in saline with DMSO (DR rats) twice daily. The groups received the corresponding treatment at 9:00 and 18:00 h during 21days prior to the experiments.

Bioassay

Rats were anesthetized with diethyl ether and killed by exsanguination. The blood was collected and kept in a sterile glass tube for further analysis. The thoracic aorta was immediately excised and placed in a Krebs' solution of the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1 and carefully cleaned from adhering fat and connective tissue. The aorta was cut into transverse rings (3–4 mm), which were then mounted on the triangular stirrups and suspended under 2 g resting tension in organ baths (Radnoti, Monrovia Ca, USA) containing 50 ml Kreb's solution and maintained at 37 °C, pH 7.4, gassed with 95% O_2 and 5% CO_2. 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 conducting the experiments, during which time the resting tension was readjusted to 2 g as required. The aortic rings were submaximally contracted with 1 μM phenylephrine (Phe). The functional presence of endothelium was verified by the ability of 1 μM Ach to induce relaxation, and an endothelium-denuded preparation was used to confirm the involvement of NO with sodium nitroprusside (SNP, 10 nM).

The contractile responses of aortic rings were expressed in percentages of change respect to their own maximum tension to 1 μM Phe. Vascular responses to the vasodilator acethylcholine (Ach) were reported in the percentage of reduction in tension (remaining contraction) compared to 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. The values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Endothelial dysfunction is notoriously associated with decreased NO bioavailability, which is due to impaired NO production by the endothelium and/or increased inactivation of NO by ROS (Sedeek *et al*., 2012; Witting *et al*., 2007). This research was designed to study the relationship between diabetes-induced hyperglycemia and endothelium-mediated arterial function in rats treated with MBE and with the antioxidant phenolic compound synthesized by *Aristotelia chilensis*, rutin.

Fasting plasma glucose levels of diabetic control rats reached as high as 287.4 ± 9 mg/dl ($P < 0.001$) compared to normoglycaemic control (group I) rats (85 ± 24 mg/dl) during the experimental period (21 days) (Table Nº 1). Chemically induction of diabetes with alloxan or streptozotocin is associated with the characteristic loss of body weight, which is due to the increased muscle wasting (Kelleher *et al*., 2010), and due to the loss of tissue, proteins and muscle dysfunction (Newsholme *et al*., 2012). Oral administration of MBE at dose level of 100 mg/day improved the body weight in diabetic rats (Table Nº 1). The increment in body weight of diabetic rats treated with the extract may be due to proper glycemic control (Mudaliar and Edelman, 2001).

Rutin, by oral gavage administration in diabetic rats produced a 32.7 ± 11% fall in fasting plasma glucose ($P < 0.01$), when compared to the ALX-diabetic control, similar to the result described by Fernandes *et al*. (2010) and Kamalakkannan and Prince (2006a) using streptozotocin (STZ)-induced diabetic rats. Figure Nº 1 shows serum glucose concentrations in ALX-rats treated daily during 3 weeks with MBE and rutin. Blood glucose reaches its lowest level approximately 4 - 6 days after both MBE and Rutin treatment.

In ALX-diabetic rats, MBE administration reduced plasma levels of glucose by 41,6% ($P < 0.001$), cholesterol by 38,6% ($P < 0.001$), LDL by 66% (0.001) and triglycerides by 25% ($P < 0.01$). It is important to notice that the reduction of total cholesterol concentration in diabetic rats treated with MBE was due to the reduction of low density lipoprotein (LDL) and not attributed to changes in high-density lipoprotein (HDL) concentration. High levels of LDL cholesterol in the blood have been linked to coronary heart disease (Mahdy *et al*., 2012). The hypoglycaemic effect is probably due to the presence of anthocyanins (Rojo *et al*., 2012), quercetin (Vessal *et al*., 2003) and rutin (Kamalakkannan and Prince, 2006b) in the MBE (Céspedes *et al*., 2010).

Table Nº 1
Effect of *Aristotelia chilensis* on some biological parameters of normal, DC and DMBE rats

	Normal	DC	DMBE
TC (mg/dl)	49 ± 13	88 ± 11**	54 ± 6*
LDL-C (mg/dl)	11 ± 6	33 ± 13**	11 ± 5**
HDL-C (mg/dl)	38 ± 8	43 ± 9 n.s.	38 ± 6 n.s.
TG (mg/dl)	77 ± 12	76 ± 8 n.s.	57 ± 6*
FPG (mg/dl)	85 ± 24	287 ± 9.2 **	168 ± 18**
IBW (g)	220 ± 21	228 ± 11 n.s.	218 ± 14n.s.
FBW (g)	234 ± 15	184 ± 9*	214 ± 11*

TC = total cholesterol; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; TG = triglycerides; FPG = fasting plasma glucose; IBW = initial body weight; FBW = final body weight of the animals from normal, DC and DMBE. All the results were expressed as mean ± DS, n = 5 animal per group. Significantly different from diabetic rats, *$P < 0.01$, **$P < 0.001$, n.s. non significancy.

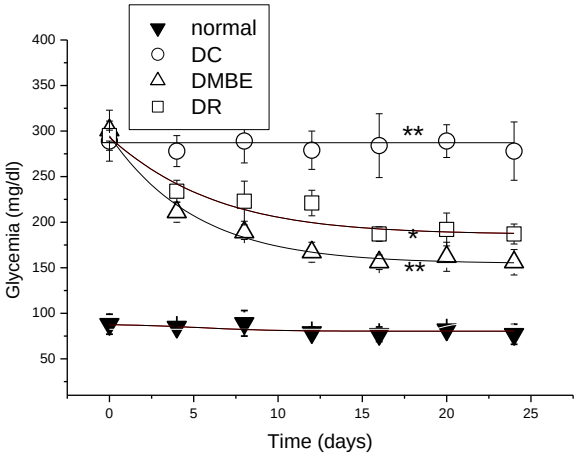

Figure Nº 1
Serum glucose concentrations in normal, DC, and ALX-rats treated daily during 3 weeks with MBE and rutin. The values represent the mean ± S.E.M. of 7 (with triplicates) animals. Significance: *$P < 0.005$; **$P < 0.001$. Student's t-test.

The hydroethanol extracts of maqui and Rutin showed an interesting protective effect on the degenerative response of endothelium under a diabetic condition. Figure Nº 2. depicts the dose-response curves induced by 10^{-9} to 10^{-5} mol/L Phe in aortic rings of the normal rats, diabetic control and rats treated with MBE and Rutin. Rings from DC-rats, with and without endothelium, elicited a higher maximal contractile response to Phe, compared with DC- rats, which was reduced by MBE and Rutin ($p < 0,001$ and $p < 0,01$ respectively).

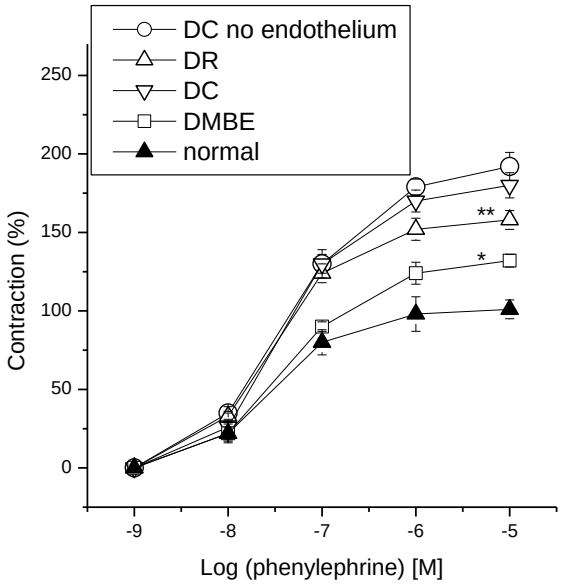

Figure Nº 2
Dose-response curves induced by 10^{-9} to 10^{-5} mol/L Phe in aortic rings of the normal rats, DC, DR and DMBE rats. The values represent the mean ± S.E.M. of 6 (with tri-plicates) animals. Significance: ****P* **< 0.01; *****P* **< 0.001. Student's t-test.

The increment in the contractile responses after Phe treatment in aortic rings from alloxan-diabetic rats is, at least in part, due to the decreased basal NO bioavailability generated by hyperglycemic condition (Olukman *et al*., 2010; Kolluru *et al*., 2012), because some findings suggest an increased negative endothelial modulation by NO as a compensatory mechanism to slow the development of tension produced by Phe (Padilha *et al*., 2008). This presumption is supported by our observations in endothelium denuded rings of DC (Figure Nº 3), where the Phe-induced contraction is greater than in the intact rings, similar when the rings were incubated in the presence of L-NAME. The same figure also shows that Phe-induced contraction in MBE and DR rings is increased in the presence of L-NAME and rings without endothelium. Therefore, the mediator of this endothelial depression of Phe sensitivity appears to be the absence of NO. The mechanisms responsible for an increased contractile response of diabetic arteries to vasoconstrictor agonists are not completely understood, although the alterations of the 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), and stimulation of smooth muscle cells with an agent acting on α1-adrenoceptors can lead to increased NO synthesis (Dora *et al*., 2000).

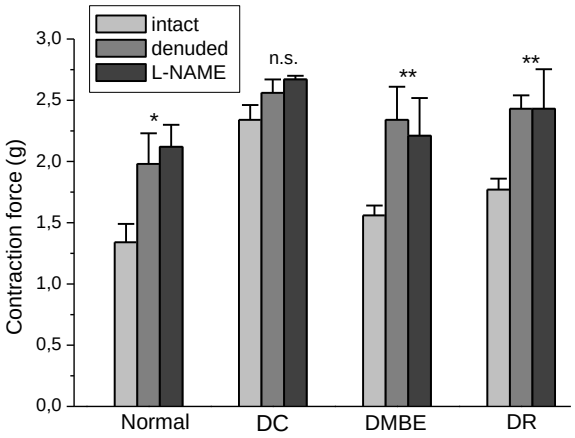

Figure Nº 3
Maximal force developed of precontracted intact, denuded and L-NAME exposed rings of normal, DC, DMBE and DR rats in response to 1µM phenylephrine. Values represent the mean ± S.E.M. of 5 animals with duplicates. Significance (denuded and L-NAME vs intact): ****P* **< 0.01; *****P* **< 0.005. Student's t-test.

The main part of the relaxing effect of Ach in aortic rings of rats depends on NO synthesis (Cachofeiro and Nasjletti, 1991); therefore, we determined the response of precontracted aortic rings of DC and DMBE rats on the vasorelaxation induced by Ach. Reactivity to Ach in aortas from DC rats after 21 days showed significant (*P* < 0,001) decreased endothelium-dependent relaxations compared to the aortas of normal rats (Figure Nº 4). However, Ach-induced relaxation of the aortic rings in alloxan-diabetic rats treated with MBE and Rutin was significantly greater (P < 0.001 and 0.01 respectively) compared to the aortas of DC.

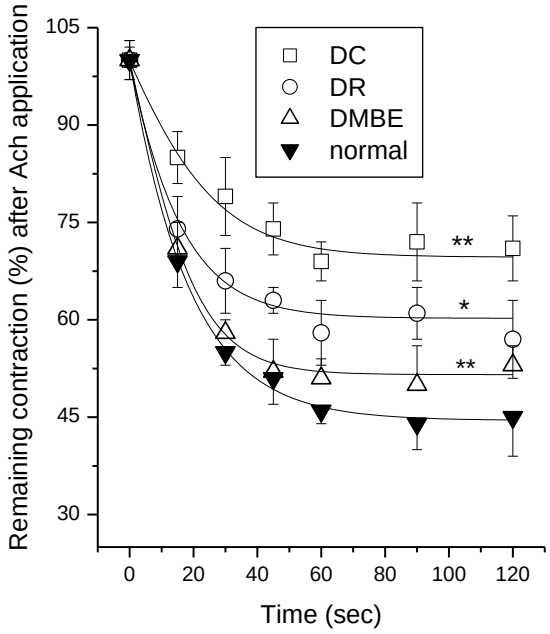

Figure Nº 4
Remaining contraction after addition of 1 µM acetylcholine on Phe-precontracted DC, DR, DMBE compared to normal aortic rings. The values represent the mean ± S.E.M. of 8 (with duplicates) animals. Significance: (DC vsus normal; DMBE and DR vsus DC) ****P* **< 0.01; *****P* **< 0.001. Student's t-test.

We do not know if the relaxing response in DMBE and DR is due to the increased availability of nitric oxide due to the reduction of free radicals, or the reduction of these as a result of hypoglycemia, because diabetic patients have an increased level of systemic free radicals, which severely restricts the bioavailability of endothelium-derived nitric oxide (NO), and thus contributes to the development of an endothelial dysfunction (Brinkmann *et al*., 2011).

CONCLUSIONS

Results show that the hydroalcoholic extract of maqui berry and Rutin, after 3 weeks administration *in vivo*, have a significant effect in lowering glucose, improving endothelium-dependent relaxation and vascular contraction in alloxan-induced diabetes, possibly by the stimulation of the nitric oxide pathway. The results also reveal that chronic *in vivo* treatment of maqui extract prevents dyslipidemia in alx-rats.

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