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Pharmacological Studies on Hypotensive, Diuretic and Vasodilator Activities of Chrysin Glucoside from *Calycotome villosa* in Rats

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The present study was undertaken in normotensive anaesthetized male rats that received a continuous perfusion of a chrysin glucoside isolated from the flowers and leaves of *Calycotome villosa* subsp *intermedia* at a dose of 2.5 mg/kg, or furosemide (control diuretic) at a dose of 0.5 mg/kg. Compared with the control rats receiving NaCl (0.9%), the urine flow, glomerular filtration and electrolyte excretion (Na^+, K^+) increased significantly in rats treated with chrysin glucoside ($p < 0.001$). A similar effect was observed in the rats perfused with furosemide. Intravenous injections of bolus doses (1–3 mg/kg) of the chrysin glucoside to anaesthetized rats elicited an immediate and dose-dependent decrease in mean arterial blood pressure (MABP). Pretreatment of the rats with the nitric oxide synthase inhibitor, L-NOArg (10 mg/kg), reduced partially, but significantly ($p < 0.01$), the maximal decrease in MABP elicited by chrysin glucoside. In the rat isolated aorta preparation, chrysin glucoside (10–100 μM) inhibited in a concentration-dependent manner the noradrenaline (1 μM) induced contractions (IC_{50} = 52 μM). This relaxant activity of chrysin glucoside was significantly reduced by incubation of the endothelium-intact rings with L-NOArg (100 μM), (80 ± 4.7% vs 48 ± 5.06% in the absence of L-NOArg). In conclusion, these results demonstrate a diuretic and hypotensive action of a chrysin glucoside from *Calycotome villosa* in anaesthetized rats and indicating an action on renal function, and an active vascular relaxation mediated partially through nitric oxide release. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: chrysin glucoside; *Calycotome villosa*; diuretic; hypotensive; vasorelaxant; endothelial dependent; rats.

INTRODUCTION

Calycotome villosa (Poiret) Link subsp. *intermedia* (C.Presl) Quezel and Santa belongs to the Papillionacea family. It is an erect shrub that can grow up to 2 m tall, especially in the north of Africa and Spain (Greuter *et al.*, 1989). The flowers are yellow and their flowering period is during the spring. The first chemical investigations of this species have led to the isolation and structural elucidation of alkaloids from the seeds (El Antri *et al.*, 2004a, El Antri *et al.*, 2004b) and flavonoid glycosides from the flowers and leaves (El Antri *et al.*, 2004c).

Flavonoids comprise a large group of naturally existing polyphenolic compounds widely distributed throughout the plant kingdom (Bohm, 1998; Bors *et al.*, 2001). They exhibit various biological effects such as inhibiting platelet aggregation, scavenging free radicals, preventing cell proliferation and reducing levels of low-density lipoproteins in plasma (Middleton, 1984; Formica and Regelson, 1995). In addition, these compounds are reported to modulate vascular tone; different studies (Hertog *et al.*, 1993; Knekt *et al.*, 1996) revealed an inverse association between flavonoid intake and the occurrence of cardiovascular diseases. However, the chemical compounds identified in *Calycotome villosa* have not been tested for their pharmacological properties to ascertain their potential therapeutic efficacy.

Thus, the purpose of the present study was to investigate the vasodilator activity of the chrysin glucoside to characterize its pharmacological effect on arterial blood pressure, and the renal excretion of water, sodium and potassium in anaesthetized rats.

MATERIALS AND METHODS

Collection of the plant and extraction of the flavone. The aerial parts of *Calycotome villosa* subsp. *intermedia* were collected in Zrireg valley, plateau of Tazzeka, area of Taza, Morocco. Authentic simples were identified at the Department of Biology, Faculty of Science, Sidi Mohamed Ben Abdellah University Fès, Morocco, where a voucher specimen was deposited (LB134). The plant was then dried and sliced. Afterwards, 30 g of the dried plant was first extracted with hexane for 24 h in order to eliminate the lipids and other fat content, then with MeOH for 48 h using a Soxhlet apparatus. After cooling the methanol extract, a yellow product

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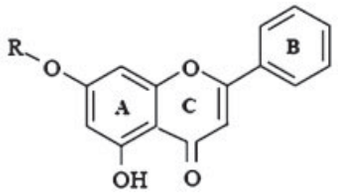

Figure 1. Structure of the chrysin glucoside.

precipitated which was filtered, dried and identified. The resultant was 7-O-(β-D-glucopyranosyl) chrysin (El Antri *et al.*, 2004c) with the chemical structure shown in Fig. 1.

Diuretic activity. Male Wistar rats weighing 200–250 g were deprived of food (12 h) but given water *ad libitum*. On the day of the experiment, they were anaesthetized with pentobarbital (1 mL/kg body weight) by the intraperitoneal route. After anaesthesia, they were placed on a heated table to maintain their body temperature at around 37 °C. A tracheotomy was performed in order to facilitate respiration. The jugular vein and the carotid artery were then cannulated with the aid of polyethylene tubing for perfusion of solutions and to collect blood samples, respectively. The bladder was then cannulated in order to collect urine samples.

Rats were subdivided into three groups: six rats in each group. The first group served as the control and received physiological salt solution (0.95% NaCl) throughout the experiment. The second group of rats received the chrysin glucoside dissolved in the vehicle solution (2.5 mg/kg). The third group of rats received a standard diuretic, furosemide, dissolved in the vehicle solution (2.5 mg/kg). Physiological solution was perfused at a rate of 50 μL/min/100 g body weight. After an equilibration period of 60 min, three urine samples were collected at 20 min time intervals. At the end of these periods, the perfusion was discontinued and replaced either by a solution containing the chrysin glucoside, or by a solution containing furosemide. Urine was collected during several periods of 20 min each for 2 h.

Blood samples were taken at the start of the control period and at the end of the experiment in order to determine the hematocrit and other parameters (creatinine, urea, sodium and potassium).

Plasma and urine sodium and potassium concentrations were determined by flame photometry (Henry *et al.*, 1974). Creatinine and urea levels in plasma and urine samples were evaluated by colorimetric methods using a spectrophotometer. Glomerular filtration rate was evaluated by the clearance of creatinine. Water, Na^+ and K^+ excretion rates are presented as mean values with their respective standard errors in μL/min and μmol/min, respectively, and the number of rats used in the experiments is indicated ($n = 6$).

***In vitro* studies in aorta rings.** Male Wistar rats weighing 200–250 g were anaesthetized with diethyl ether and killed by decapitation. The thoracic aorta was isolated rapidly and excess fat and connective tissues were removed. Segments, about 2 mm in length, were suspended between two hooks and mounted in 12.5 mL organ baths filled with a physiological salt solution (0.95% NaCl) having the following composition (mM): NaCl, 122; KCl, 5.9; $NaHCO_3$, 15; $MgCl_2$, 1.25; $CaCl_2$, 1.25; glucose, 11, as described (Ghisdal *et al.*, 1999; El Bardai *et al.*, 2001). The bath solution was maintained at 37 °C and gassed with a 95% O_2–5% CO_2 mixture. A basal tension of 20 mN was applied to the artery rings. After an equilibration period, each preparation was contracted by changing the physiological solution in the bath to a depolarizing 100 mM KCl solution (composition in mM: NaCl, 27; KCl, 100; $NaHCO_3$, 15; $MgCl_2$, 1.25; $CaCl_2$, 1.25; glucose, 11). The endothelium integrity was tested by measuring the relaxation evoked by acetylcholine (1 μM). After washing, and a 60 min recuperation time, a test contraction was evoked either by changing the solution in the bath to the K^+ solution or by adding noradrenaline (1 μM) to the physiological salt solution (0.95% NaCl) in the bath. The effect of the chrysin glucoside was tested by cumulative addition into the bath when contraction had reached a plateau. The change in the contractile tension evoked by the chrysin glucoside was compared with the effect of the addition of the same volume of the vehicle (DMSO) into the bath. In some experiments, preparations were preincubated for 30 min with the NO synthase inhibitor N-ω-nitro-L-arginine (L-NOArg, 100 μM).

Measurement of blood pressure in anaesthetized rats. Male Wistar rats (200–250 g) were used and anaesthetized with an intraperitonial injection of pentobarbital (1 mL/kg body weight). The trachea was exposed and cannulated to facilitate spontaneous respiration. Two catheters (PE-50) were implanted in the left carotid artery and the right jugular vein for recording arterial blood pressure and for drug administration, respectively, as described previously (Ghayur and Gilani, 2005).

The rats were injected with heparinized saline (0.1 mL, 50 IU/mL) to prevent blood clotting. The arterial catheter was filled with heparinized saline, which was connected to a pressure transducer (D.C. Driver amplifier) and recorded on a Grass model 79 D polygraph. The mean arterial blood pressure was calculated as the diastolic blood pressure plus one-third of the pulse width. After 20 min of equilibration, each animal received a series of increasing bolus doses of the chrysin glucoside of *Calycotome villosa* (1, 2, 2.5 and 3 mg/kg). Arterial blood pressure was allowed to return to baseline level before the subsequent dose was administered. Changes in blood pressure were expressed as the difference between the steady state value before drug injection and the peak value recorded after drug injection.

Drugs. All standard compounds used in this study were obtained from Sigma. The chrysin glucoside was dissolved in a small amount of DMSO (devoid of any effect) and the volume was made up by adding physiological salt solution (0.95% NaCl).

Statistical analysis. The results were expressed as mean ± SEM. Comparisons were made by Student's *t*-test or analysis of variance (ANOVA) followed by Bonferroni test. In all analyses, $p < 0.05$ was considered significant. pD_2 values (–log of the concentration producing 50% inhibition, IC_{50} were calculated by non-linear regression of the mean data (Prism, Graph Pad).

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RESULTS

Diuretic activity

Effect on the diuresis and electrolyte excretion. The intravenous perfusion of chrysin glucoside (2.5 mg/kg) increased the urinary flow from 8.5 ± 0.5 to 26.4 ± 1.6 µL/min after 1 h of treatment ($p < 0.05$). Two hours after beginning the perfusion, the chrysin glucoside increased the urine output from 8.5 ± 0.5 to 50.5 ± 16.58 µL/min ($p < 0.001$), while urine output in control rats was 5.2 ± 0.4 before the period of treatment and 7.5 ± 0.7 µl/min after 3 h (Fig. 2A). As expected, the urine output increased significantly following perfusion of furosemide at a dose of 0.5 mg/kg ($p < 0.001$).

The effects of the chrysin glucoside and furosemide on sodium ($U_{Na+}V$) and potassium urinary excretion ($U_{K+}V$) are shown in Fig. 2B and Fig. 2C, respectively. The urinary excretion of sodium increased significantly following the perfusion of chrysin glucoside at a dose of 2.5 mg/kg (from 24.18 ± 2.04 to 75.53 ± 1.14 µmol/min), while in control rats the Na^+ excretion rate increased from 25.83 ± 2.26 to 32.02 ± 0.23 µmol/min ($p < 0.001$). On the other hand, the urinary excretion of potassium increased moderately but significantly following the perfusion of chrysin glucoside from 13.11 ± 0.84 to 24.26 ± 1.87 µmol/min. In control rats K^+ excretion rate was 11.04 ± 0.39 and 13.97 ± 0.32 µmol/min ($p < 0.001$), during the same periods. A significant increase of sodium and potassium appeared after the perfusion of furosemide (24.68 ± 0.43 to 87.18 ± 0.63 µmol/min and 11.15 ± 0.67 to 37.21 ± 1.16 µmol/min, respectively, $p < 0.001$).

However, it is noticed that there was no significant difference in the effect of chrysin glucoside and

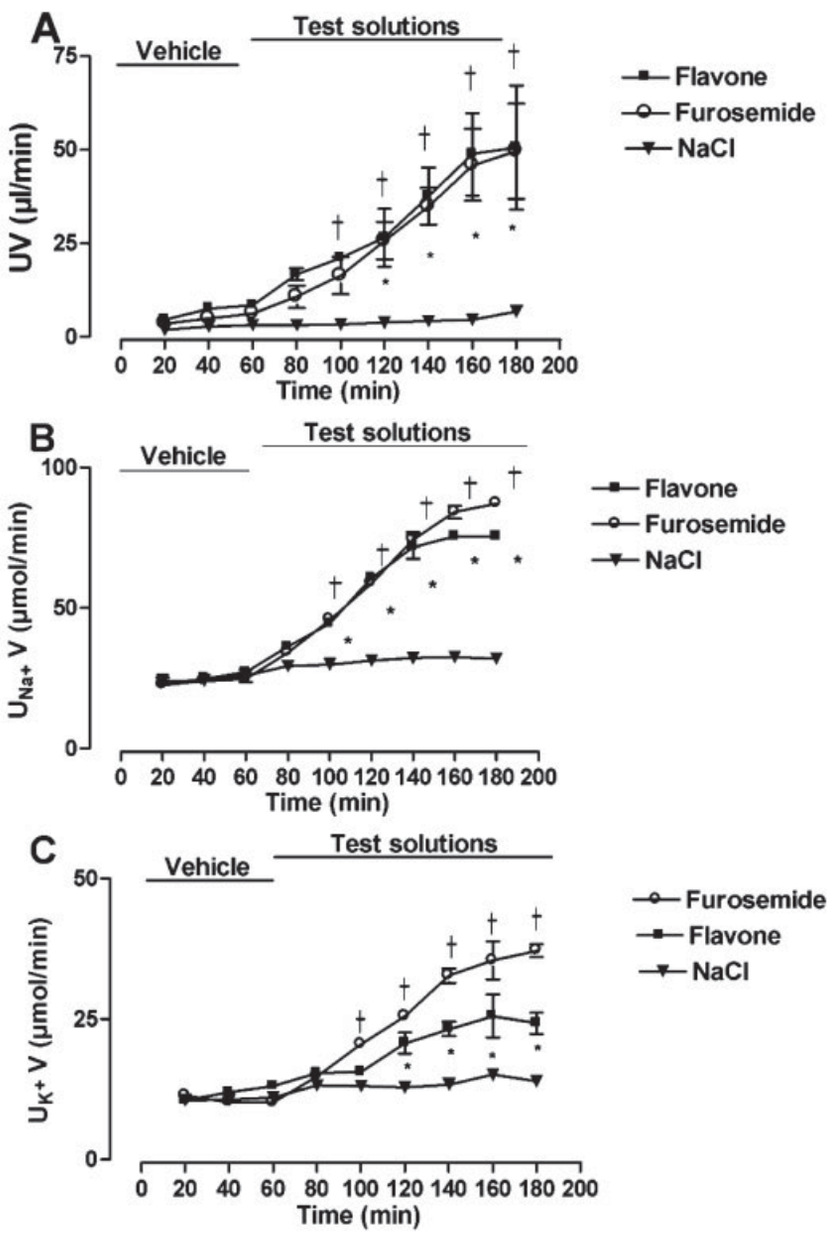

Figure 2. (A) Effects of intravenous perfusion of the chrysin glucoside on urine output (UV) (µl/min). (B) Urinary excretion of sodium (UNa^+V (µmol/min). (C) Urinary excretion of potassium (UK^+V) (µmol/min). ▼: Control (NaCl 0.9%); ■: Furosemide (0.5 mg/kg); ○: Flavone (2.5 mg/kg). Data are mean ± SEM of six rats in each group; *: significant difference between control and flavone-treated rats ($p < 0.05$); †: significant difference between control and furosemide-treated rats ($p < 0.05$). Test used was analysis of variance (ANOVA) followed by Bonferroni test.

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Table 1. Effects of the perfusion of chrysin glucoside on the plasma concentration of sodium, potassium and urea (mmol/L) on the glomerular filtration rate (mL/min) and hematocrit (Hct) in plasma samples before (T_i) and after (T_f) treatment

	Sodium		Potassium		Glomerular filtration rate		Urea		Hct (%)	
Treatment	T_i	T_f	T_i	T_f	T_i	T_f	T_i	T_f	T_i	T_f
Control (NaCl 0.9%)	142 ± 2.1	143 ± 1.9	4.3 ± 0.5	4.4 ± 0.4	0.19 ± 0.07	0.24 ± 0.1	7.3 ± 0.5	7.2 ± 0.7	45 ± 1.2	42 ± 1.5
Flavone (2.5 mg/kg)	145 ± 1.4	143 ± 2.9	4.2 ± 0.3	4.1 ± 0.8	0.24 ± 0.05	1.31 ± 0.2[a]	7.2 ± 0.3	7.4 ± 1.1	44 ± 0.6	43 ± 0.8
Furosemide (0.5 mg/kg)	146 ± 1.9	144 ± 2.5	4.1 ± 0.4	2.9 ± 0.4[a]	0.22 ± 0.02	1.56 ± 0.2[a]	7.3 ± 0.4	7.2 ± 0.9	46 ± 0.6	44 ± 2.2

Values are expressed as mean ± SEM from six rats.

[a] $p < 0.05$ when compared with the control group which received physiological solution (NaCl 0.9%). Test used was Student's *t*-test.

furosemide on urine output and the urinary excretion of sodium ($p > 0.05$). On the other hand, there was a less effect of chrysin glucoside on the excretion of potassium compared to that of furosemide ($p < 0.01$).

Effects on glomerular filtration rate. Table 1 shows the values of the glomerular filtration rate (GFR) before and after treatment. After the intravenous perfusion of chrysin glucoside (2.5 mg/kg), GFR was significantly increased from 0.24 ± 0.05 before injection to 1.31 ± 0.17 mL/min after injection of the chrysin glucoside ($p < 0.001$). Furosemide produced a similar increase in GFR. The perfusion of physiological salt solution (0.95% NaCl) did not cause any significant change in GFR (Table 1).

Effects on electrolytes, urea and hematocrit in plasma samples. No remarkable changes were noticed in plasma electrolytes (Na^+, K^+) concentration after intravenous perfusion of chrysin glucoside at the dose used, while furosemide induced a moderate decrease of plasma K^+ levels from 4.1 ± 0.4 to 2.9 ± 0.4 mmol/L ($p < 0.05$) (Table 1). Neither the chrysin glucoside nor furosemide exerted a significant effect on plasma urea and hematocrit (Table 1).

Effects on rat isolated aorta. The vascular effect of the chrysin glucoside was investigated in isolated aorta precontracted with noradrenaline or high K^+ solution. The addition of chrysin glucoside to the bath solution when contraction had reached a plateau, caused a pronounced concentration-dependent relaxation (Fig. 3).

The chrysin glucoside showed a greater relaxant effect in noradrenaline-contracted aortic rings compared with K^+-contracted arterial preparations. The concentration producing 50% inhibition of the contractile response to noradrenaline (IC_{50}) was 52 μM. At the highest concentration used (100 μM), the chrysin glucoside only relaxed K^+ contractions by 27%.

Pretreatment of tissue with L-NOArg (30 min) attenuated the inhibitory effect of the chrysin glucoside against noradrenaline-induced contraction. In the absence of the L-NOArg, 100 μM of the chrysin glucoside relaxed noradrenaline-contraction by 80%. This effect was significantly reduced after pre-incubation of the aortic rings with L-NOArg, the chrysin glucoside relaxing the contraction by 51% only ($p < 0.05$) vs in the absence of L-NOArg.

Effect on blood pressure. The effect of chrysin glucoside was tested on arterial blood pressure in anaesthetized normotensive rats. The baseline values of the mean arterial blood pressure was 88.66 ± 5.6 mm Hg; $n = 6$). The intravenous administration (1–3 mg/kg) of chrysin glucoside caused a rapid dose-dependent fall in arterial blood pressure (Figs 4, 5).

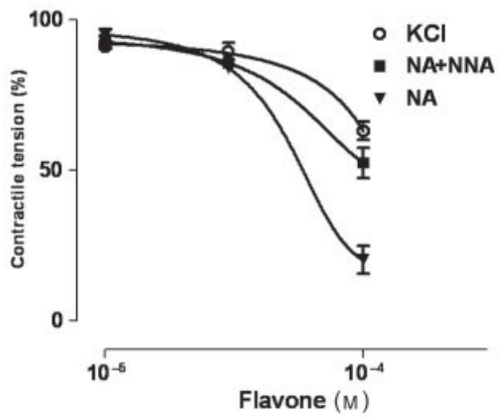

Figure 3. Effects of chrysin glucoside on the contraction of rat aorta rings precontracted with high-K^+ solution or with noradrenaline in the absence and presence of N-nitro-L-arginine (L-NOArg, 100 μM). Pretreatment of tissue with NNA reduced the relaxant effect significantly ($p < 0.001$). Relaxation induced by cumulative additions of chrysin glucoside is expressed as a percentage of contraction after precontraction with K^+ (100 mM) or noradrenaline (1 μM). Each point represents mean ± SEM of 5–6 rings.

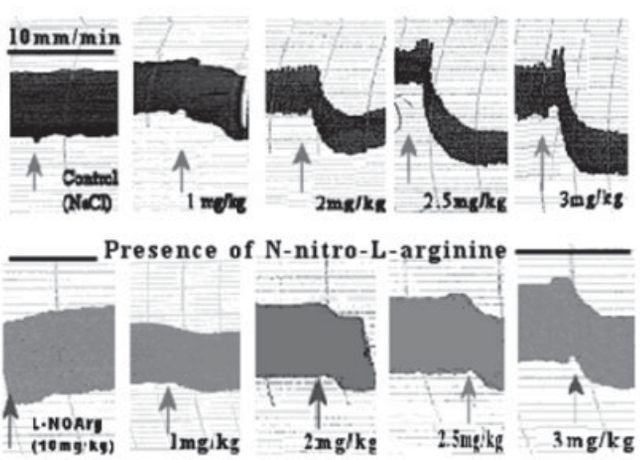

Figure 4. Typical recordings of alterations in the arterial blood pressure (BP) of anaesthetized rats after intravenous administration of different doses of chrysin glucoside in the absence (upper traces) and presence (lower traces) of N-nitro-L-arginine (L-NOArg, 10 mg/kg).

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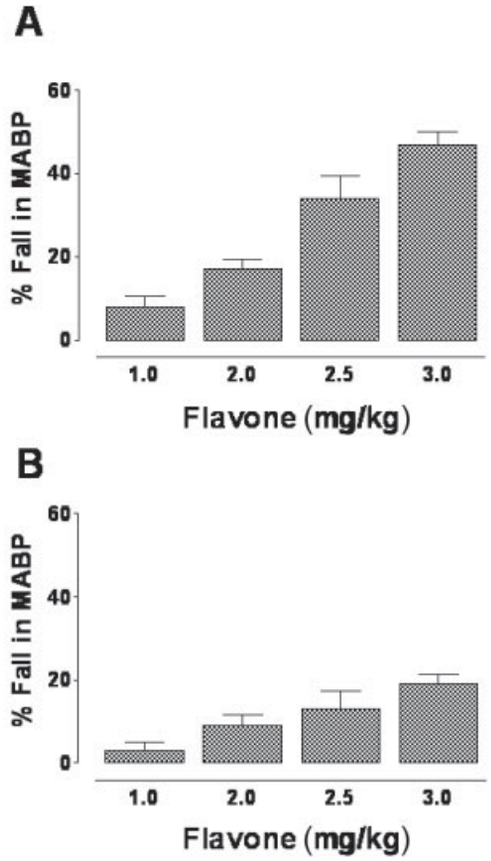

Figure 5. Dose-dependent hypotensive effect of chrysin glucoside in anaesthetized rats. Data are expressed as mean ± SEM ($n = 6$) in the absence (A) and presence of N-nitro-L-arginine (10 mg/kg). Pretreatment with N-nitro-L-arginine reversed the fall in blood pressure significantly ($p < 0.001$).

In order to determine whether this effect could be related to the control of blood pressure by NO, rats were first treated with L-NOARG (10 mg/kg) 20 min before injection of the chrysin glucoside. Intravenous administration of L-NOArg increased the blood pressure by 33%. In L-NOArg treated rats, the hypotensive effect of chrysin glucoside was significantly reduced (Fig. 5B).

DISCUSSION

The results of the present study demonstrate that the intravenous perfusion of chrysin glucoside to anesthetized rats at dose of 2.5 mg/kg caused a significant increase in urinary and electrolyte excretion. This effect was similar to that of furosemide, which was used as a positive control with known saluretic and diuretic effects (Leuschener, 1995). Furthermore, the intravenous injection of chrysin glucoside to anesthetized rats at doses ranging from 1–3 mg/kg elicited a dose-dependent decrease in arterial blood pressure. Pretreatment of animals with N-nitro-L-arginine significantly reduced the hypotensive effect of chrysin glucoside, which suggests that the blood pressure lowering effect of the chrysin glucoside is partially dependent on the activity of the NO synthase.

The *in vitro* study showed that vasorelaxation elicited by the chrysin glucoside in isolated aorta was partly dependent on the presence of a functional endothelium (data not shown) and endogenous NO generation, because inhibition of NO synthase or the removal of endothelium decreased the vasorelaxant effect of the chrysin glucoside. Similarly, it caused inhibition of high K^+-induced contractions at relatively high doses which suggests some involvement of an endothelium independent effect possibly mediated through calcium channel blockade (Ghayur and Gilani, 2005).

Several groups of flavonoids have been shown to possess vasorelaxant properties (Duarte *et al.*, 1993; Duarte *et al.*, 1994; Herrera *et al.*, 1996; Chen and Pace-Asciak, 1996; Chan *et al.*, 2000; Duarte *et al.*, 2001). Chrysin has been reported to inhibit noradrenaline and K^+-evoked contractions in rat aorta (Chan *et al.*, 2000; Chen and Pace-Asciak, 1996). As observed with chrysin glucoside, it was a more potent inhibitor of noradrenaline-evoked contractions than of K^+-evoked contractions (Chan *et al.*, 2000). However, in contrast to the effect of chrysin glucoside, the relaxation evoked by chrysin was not affected by NOS inhibition (Chen and Pace-Asciak, 1996; Chan *et al.*, 2000).

The chrysin glucoside increased urine output and electrolyte excretion. It is possible that its diuretic effect is mediated by inhibiting the tubular reabsorption of water and accompanying anions, which was accompanied by an increase in GFR through a mechanism qualitatively similar to that of furosemide and more than one mechanism seems to be involved. Under these conditions, it was shown that the chrysin glucoside was able to induce diuretic, natriuretic and kaliuretic responses which may be complementing the hypotensive activity found on anesthetized rats. These results suggest different mechanisms of action, such as a direct effect of the chrysin glucoside on arterial blood pressure which could affect the glomerular filtration rate (Bevevino *et al.*, 1994) or by decreasing renal perfusion pressure (Bevevino and Mello Aires, 1994). However, further studies are required to determine the precise location of action in the nephron and the underlying mechanisms involved with Na^+, K^+ and water excretion.

A third possibility is that the diuresis possessed by chrysin glucoside may be mediated by an increase in renal blood flow, and then, in glomerular filtration, such an effect would result from direct vasodilatation and then hypotension. The released nitric oxide from vascular endothelial cells appears partially involved in the aortic relaxation and in turn in the mediation of chrysin glucoside induced hypotension.

In summary, the chrysin glucoside of *Calycotome villosa* subsp. *intermedia* is a potent compound for its hypotensive and diuretic activities and exhibits vasodilation mediated through endothelium dependent and independent mechanisms and further studies are required to understand the precise mode of action.

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