



Inorganic Ions in *Leonotis Leonurus* Extract Do Not Explain Changes in Isolated Wistar Rat Heart Function

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Abstract: *Leonotis leonurus* (*L. leonurus*) R. Br. (Lamiaceae) is used by healers in South Africa. The present study was conducted to determine if the *L. leonurus* effect on isolated rat heart is due to ionic changes in the perfusion fluid. Isolated rat hearts were perfused after Langendorff at 74 mmHg with modified Krebs–Henseleit buffer (K-H) and three test solutions: LI (2 mg/ml dried crude aqueous leaf extract in K-H), HighK (K-H with $[K^+] = [K^-]$ in LI), and HighMgK (K-H with both $[K^+]$ and $[Mg^{2+}] =$ that in LI). $[Na^+]$, $[Cl^-]$, $[Ca^{2+}]$ and $[PO_4^{3-}]$ did not change significantly in LI but the $[K^+]$ and $[Mg^{2+}]$ were increased significantly. LI stopped some hearts completely and reduced all parameters excluding left ventricular end diastolic pressure (LVEDP) while other hearts became erratic showing increased LVEDP and coronary flow (Q), with decreased heart rate (HR), left ventricular developed pressure (LVDP) and cardiac work (CW). HighK had no significant effect on any parameters. HighMgK reduced LVESP, LVEDP, LVDP, HR, CW and Q vs. control but less so than LI. Neither the HighK, nor the HighMgK mimicked the changes observed with LI. The effects of LI are thus not due to changes in ionic composition of the perfusion fluid.

Keywords: *Leonotis leonurus*; Langendorff perfusion; potassium; magnesium; ionic composition.

Introduction

Leonotis leonurus R. Br. (Lamiaceae) is also known in Afrikaans as *duiwelstabak*, *koppiesdagga*, *rooidagga*, *wildedagga*; in English as Cape hemp, lion's ear, *wild dagga*; and in Zulu as *imunyamunya*, *umunyane*, *umunyane-omncane* (Hutchings et al. 1996; van Wyk et al. 2000). *Dagga* is the South African colloquial term for marijuana. It is also known as Minaret flower (Turkey) (Watt and Breyer-Brandwijk 1962). Scientific publications on the biological effects of this plant are scarce although numerous authors reported the use of *L. leonurus* as a medicinal plant for a wide range of conditions including epilepsy, coughs, colds and influenza, bronchial complaints, hypertension, headaches, asthma, as an anthelmintic, haemorrhoids (piles), eczema, skin rashes, boils, viral hepatitis, insect bites or stings, and dysentery. It is also reportedly used as a strong purgative, an enemagogue, and a

general tonic (Batten and Bokelmann 1966; Ferreira et al. 1996; Hutchings et al. 1996; Pijper 1919; Roberts 1990; Scott et al. 2004; van Wyk et al. 2000; Watt 1967; Watt and Breyer-Brandwijk 1962). Leaves and flowers were chewed by the Xhosa to relieve epigastric pain while root sap was used to clear sinuses (Van Wyk and Gericke 2000). It is also used as a traditional medicine to treat menstrual disorders in South Africa (Scott et al. 2004). According to Van Wyk et al. (2000) there are some doubts about claims in early reports (Forbes 1986; Smith 1966) that it was smoked as a substitute for marijuana (*Cannabis sativa L*) since it is only mildly narcotic (Watt and Breyer-Brandwijk 1962).

Controlled scientific studies on the biological effects of extracts from this plant found that the water extract of *L. leonurus* had anticonvulsant activity and might be acting through non-specific mechanisms, since it

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affects both gabaergic and glutaminergic systems (Bienvenu *et al*, 2007). High performance liquid chromatography (HPLC) and phytochemical tests showed the presence of alkaloids, saponins and tannins in the extract (Scott *et al*. 2004). Maphosa *et al*. (2010) found *in vitro* evidence for the justification of using

this plant for the treatment of gastrointestinal helminthosis. Subsequent *in vivo* studies prompted a warning that the plant extracts can be toxic and may even cause deaths (Maphosa and Adedapo 2008). Khan (2007) reported effects on the isolated heart of the Wistar rat.

Table 1: % Change due to 3 min constant flow perfusion of LI (*L. leonurus*) in K-H. [mean±SD(n)] LI: *L. leonurus*; LVDP: Left ventricular developed pressure; LVSP: Left ventricular systolic pressure; HR: Heart rate; CW: Cardiac work; PPerf : Perfusion pressure. *: significant difference from control (P<0.05) (Khan 2007)

[LI] mg/ml	% change in LVDP	% change in LVSP	% change in HR	% Change in CW	% Change in PPerf
0.0	2.65±1.80(6)	0.03±0.63(6)	-0.05±1.23(6)	-0.56±0.82(6)	0.63±1.20(6)
0.1	-10.86± 24.40(5)	5.37±2.98(5) *	-2.47±2.07(5)	2.91±4.81(5)	-1.61±2.41(5)
0.5	-9.29±13.29(8)	7.35±4.90(8)*	-2.37±2.50(8)	5.18±3.23(8)*	-4.44±5.72(8)*
1.0	37.79±82.66(7)	23.13±6.63(7)*	-28.28±4.94(7)*	-13.27±6.89(7)*	-11.45±7.74(7)*
2.0	101.50±11.74(5)	-4.17±9.15(5)	-42.71±8.02(5)*	-48.84±4.89(5)*	-15.86±12.51(5)

With the above somewhat contradictory reports in mind, and because the practice of South African traditional healers has been recognized by an Act of Law (Republic of South Africa Act number 35 of 2004, 2005), the use of traditional remedies must be more comprehensively investigated.

Skrzypiec-Spring *et al*. (2007) affirmed that the isolated mammalian heart preparation is a very useful tool in modern cardiovascular and pharmacological research and that the method brought important advances in many areas in the previous decade including ischemia-reperfusion injury, cell-based therapy and donor heart preservation for transplant. In the classical Langendorff perfusion model the heart is rapidly excised and the aorta is attached to a cannula through which perfusion fluid is supplied to the myocardium.

Constituents of the final experimental perfusion fluid can be placed in three categories: a) the inorganic chemicals and glucose making up the K-H buffer, b) the inorganic ions present in the *L. leonurus* extract, and c) any other compounds present in the extract. The final function of the heart can be influenced by all three of these groups. It is well known that plants contain inorganic compounds and that the inorganic composition of the perfusion fluid can

influence cardiac contractility. The extraction methods used would result in these water soluble plant components being present in the experimental perfusion fluid. It was thus possible that the presence of these inorganic compounds in the plant extracts could have influenced the composition of the perfusion fluid sufficiently to cause an effect on the cardiovascular parameters under study. The current study therefore investigated the effect of the crude aqueous extract of this plant on the ionic composition of the perfusion fluid and its subsequent influence on the observed contractility parameters of the isolated perfused Wistar rat heart.

This study also improves the scientific basis for the use of *L. leonurus* since its health benefits have not yet been fully investigated.

Methods

L. leonurus material was sourced from Kirstenbosch Botanical Gardens, Kirstenbosch, Cape Town, South Africa, during March and the identification was confirmed by Dr. Gillian Scott, taxonomist of the South African Traditional Medicine Experimental Research Group (SATMERG), School of Pharmacy, UWC, Bellville, South Africa. A voucher

specimen (TRAD-10) was deposited at the Kirstenbosch Botanical Garden herbarium. The leaves and smaller stems were dried to constant weight in a ventilated oven at a temperature not exceeding 35°C after which it was milled and the powder passed through a sieve (850µm mesh). Boiling distilled water (200ml for each 5g powdered plant material) was added, the mixture was thoroughly stirred for 10 minutes and strained through glass wool. The filtrate was freeze-dried for 24 hours (Freeze Mobile 12SL, Virtis Company, Gardiner, New York. 12525) to yield ±1g per 5g powdered plant material. The freeze dried extract was stored at -4°C and reconstituted on the day of use in modified Krebs-Henseleit buffer (K-H) having the following composition in mM: NaCl: 118; NaHCO₃: 26.2; KCl: 4.7; KH₂PO₄: 1.2; MgSO₄: 0.4; CaCl₂: 1.2; D-glucose: 11.2. All these chemicals were analytical grade and obtained from Merck Chemicals (PTY) Ltd. (Johannesburg, South Africa).

All experiments were performed according to internationally accepted guidelines for the care and use of laboratory animals and were approved by the ethics committee of the University of the Western Cape. The sample size in all groups was six, except for the StoppedLL group that had five observations.

Wistar rats from the inbred colony of the South African Medical Research Council at Medicina, Parow Valley, South Africa, were sourced and fed on standard rat pellets (Epol, Westville, South Africa). Rats were kept under a 12h light/dark cycle and were allowed water and food *ad libitum*. On the day of the experiment, the rats were anaesthetised by intraperitoneal injection of 60 mg/kg sodium pentobarbitone (Kyron Laboratories (Pty) Ltd, Benrose, South Africa). Immediately after the abolition of deep pain reflexes the hearts were rapidly excised and mounted on a modified Langendorff perfusion system (Langendorff 1897) and perfused with a modified K-H.

Three test solutions were prepared: LI was prepared by adding *L. leonurus* extract to K-H to give a final concentration of 2 mg/ml of perfusion fluid. After the inorganic composition of the LI had been determined (see results), a HighK and a HighMgK solution were prepared.

The HighK solution contained added KCl to elevate the [K⁺] to 11.0 mM, which was equal to that found in the LI. The HighMgK solution was made by the addition of MgCl₂ to give a [Mg²⁺] of 1.7 mM as well as by adding KCl to give a [K⁺] of 11 mM in order to increase the level of these two ions to the same concentrations found in LI. Perfusion solutions were always filtered through a 0.45µm filter (Millex®, Millipore, Molsheim, France) before use to prevent particulate embolism.

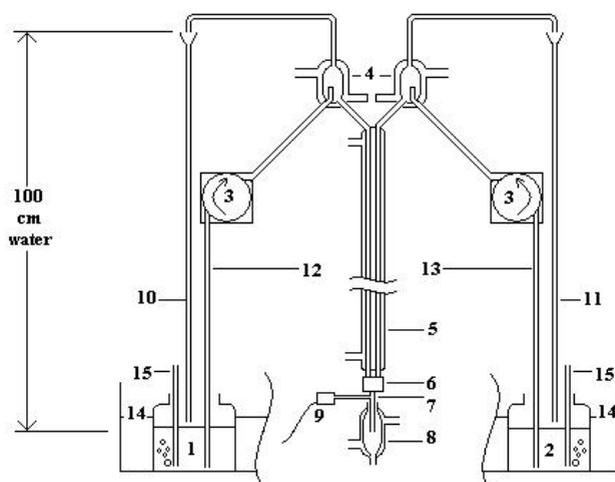


Figure 1: A modified double sided Langendorff perfusion system. (Not to scale) Water jackets maintained septal temperature at 37°C. Perfusion pressure: 100 cm Water. 1. Control perfusion solution; 2. Test perfusion solution; 3. Pump; 4. Bubble traps; 5. Perfusion lines; 6. Three way tap; 7. Aorta cannula; 8. Heart chamber; 9. Perfusion pressure transducer; 10 & 11. Return lines for overflow to the reservoirs; 12 & 13. Feed lines for the pump; 14. Water bath; 15. Bubblers.

The classical Langendorff perfusion model was modified to allow better interpretation of the results. In the modified constant pressure perfusion system (Figure 1), the perfusion fluid in each of the hydraulic channels was pumped by a dual channel Gilson Minipulse 3 peristaltic pump (LASEC, Cape Town, South Africa) to the perfusion fluid inlet of a bubble trap with an overflow that was kept 100 cm above the level of the aorta to provide the 100 cm H₂O constant perfusion pressure head. The overflow drained into a funnel and returned to the relevant fluid reservoir through silicone tubing. This

arrangement prevented fluctuations in the perfusion pressure due to the peristaltic action of the pump and minimized the risk of gas bubbles entering the aorta. The fluids in the reservoirs in the water bath were bubbled with a 96% O₂/ 4% CO₂ gas mixture (Afrox, Johannesburg, South Africa) to ensure proper oxygenation of the cardiac tissue and maintenance of the pH at 7.4.

The outflow of each bubble trap was connected by means of water jacketed plastic tubing to one of the openings of a three way stopcock. The third opening of the stopcock was connected directly to the aortic cannula. The aorta was mounted on the aortic cannula and secured with cotton thread. The aortic cannula and the isolated heart were enclosed by a water jacketed Pyrex organ chamber (Scientific Glass Blowers, Stellenbosch, South Africa). Preheated water from the water bath equipped with a temperature control unit and a circulator (Scientific, Laboratory and Scientific Equipment Company, Cape Town) surrounded the perfusion fluid reservoirs, the organ chamber, the perfusion lines and the bubble traps. This arrangement gave excellent temperature control of the perfusion system. The temperature of the heart was maintained by appropriate adjustment of the water bath temperature and monitored with a thermistor probe that was connected to a personal computer via a temperature interface (Gentronics, Cape Town, South Africa) and inserted into the right ventricle through a small incision at the origin of the pulmonary artery. Good temperature control was essential because the cardiac contractility is extremely sensitive to temperature fluctuations.

Following complete anaesthesia of the rats, the rat hearts were removed and placed in ice cold K-H buffer. After the Heart-Aorta preparation had been cleaned of excess tissue and mounted on the cannula perfusion was established by fully opening the stopcock.

An empty PVC balloon made from domestic cling wrap (Fresta Holdings Ltd., Brackenfell, South Africa) was inserted into the left ventricle through the bicuspid valve. This balloon was connected to a Deltran II pressure transducer (Utah Medical Products, West Midvale, Utah,

USA) by means of a water filled stiff walled tube and was used to monitor the left ventricular pressure. The volume of the left ventricle (preload) was adjusted by appropriate filling of this balloon with distilled water.

After the hearts were hooked up and contractions reestablished, it was perfused for 10 minutes with pure perfusion fluid to establish control values. Following this control period, the experimental solutions were administered for 5 minutes by switching to the experimental hydraulic line. After 5 minutes of perfusion with the experimental solution, the aorta was reconnected to the control buffer by switching the stopcock.

The electrical output from the transducer and temperature probe was sampled at 100Hz by an analogue - digital converter (PC-30, Eagle Electronics, Cape Town, South Africa) in a personal computer *via* an interface and software (developed in house by AP Burger). The software determined the left ventricular end-systolic pressure (LVESP) and the left ventricular end-diastolic pressure (LVEDP) from the continuously recorded left ventricular pressure. The instantaneous heart rate (HR) was automatically calculated from the duration of each cardiac pressure cycle. The left ventricular developed pressure (LVDP) was also calculated as the difference between the LVESP and the LVEDP. Cardiac Work (CW) was calculated as LVDP x HR.

Coronary perfusion (Q) (ml/min) was determined by timed collection of the effluent from the right ventricle.

Results were expressed as % change from the control values for all parameters. The actual changes in LVEDP is also reported.

Aliquots of control and test solutions were analyzed by means of an Advia 1650 Autoanalyzer (Siemens, Cape Town) and concentrations of the inorganic ions were expressed in mM.

Statistics

Data is reported as mean \pm standard deviation (number of observations) and treatment groups were compared by means of

the unpaired two tailed t-test in order to determine significance. In those comparisons where the F-test indicated that the variances differed significantly, the t-test with Welch's correction was applied. All comparisons with a $P < 0.05$ were deemed significantly different and those with $P > 0.05$ as non-significant. The slope of the linear regression lines for the changes in ionic composition with increasing *L. leonurus* in the test solutions were tested by means of the F-test for deviation from zero and the Runs test was used to test if these regression lines deviated significantly from linearity (GraphPad Prism 4, GraphPad Software, Inc San Diego, USA).

Results and Discussion

All parameters except diastolic pressure were expressed as % change in order to compensate for biological variability in the individual rats. Each heart acted as its own control and the parameter of interest was expressed as a % change relative to the control value. Actual changes in LVEDP was reported.

The average time that elapsed from the time that the thorax was opened to the time that the heart was immersed in the ice cold bubbled K-H was less than 40 seconds. It is unlikely that this would cause preconditioning since Awan et al. (1999) states that normothermic transfer times up to 3 minutes will not precondition the isolated rat heart. We also allowed adequate time for the preparation to stabilize before testing.

Ionic Composition of the Perfusion Fluid

Table 2: Ionic composition (Mean mM \pm SD) of perfusion fluid with increasing [*L. leonurus*]. (*differs significantly from control, $P < 0.05$) n=6.

[<i>L. leonurus</i>] (mg/ml)	Control 0	0.5	1.0	2.0
Sodium	141.0 \pm 5	141.5 \pm 5.0	139.8 \pm 4.9	140.5 \pm 4.6
Chloride	122.5 \pm 4.5	124.0 \pm 1.0	125.525 \pm 2.9	126.5 \pm 4.6
Calcium	1.3 \pm 0.0	1.2 \pm 0.0	1.3 \pm 0	1.3 \pm 0
Phosphate	1.25 \pm 0.03	1.24 \pm 0.01	1.33 \pm 0.08	1.35 \pm 0.03
Magnesium	0.42 \pm 0.09	0.72 \pm 0.0*	1.10 \pm 0.06*	1.71 \pm 0.04*
Potassium	6 \pm 0.1	6.5 \pm 0.4*	8.2 \pm 0.2*	10.6 \pm 0.3*

The ionic composition of the perfusion fluid after addition of *L. leonurus* is shown in Table 2. No significant change in $[\text{Na}^+]$, $[\text{Cl}^-]$, $[\text{Ca}^{2+}]$ and $[\text{PO}_4^{3-}]$ of the perfusion fluid resulted from the addition of 2 mg/ml *L. leonurus*. The slope of the regression lines for the $[\text{Mg}^{2+}]$ ($P=0.0011$) and the $[\text{K}^+]$ ($P=0.0104$) with an increase in the *L. leonurus* concentration deviated significantly from 0. These regression lines showed a significant linear increase over this concentration range as can be seen by the high R^2 for the linear regression equations for Mg^{2+} ($Y=0.615-0.6389$; $R^2 = 0.9978$) and K^+ ($Y = 2.418X-2.363$; $R^2: 0.9793$) respectively. According to the Runs test neither the regression line for the $[\text{K}^+]$ ($P=1.00$) nor that for the $[\text{Mg}^{2+}]$ ($P=1.00$) was significantly non-linear.

Maphosa and Adedapo (2008) administered high oral doses of *L. leonurus* to rats and found no significant changes in the plasma levels of sodium, potassium, and chloride. Khan (2007) did not determine the concentration of the perfusion fluid after addition of *L. leonurus* extract (0.0 - 2.0 mg/ml). We determined the $[\text{Na}^+]$, $[\text{Cl}^-]$, $[\text{Ca}^{2+}]$ and $[\text{PO}_4^{3-}]$ of the perfusion fluid (Table 2) in the concentration range of *L. leonurus* tested by Khan (2007) and found that it did not change significantly. Therefore, it was considered unlikely that the observed effect of the extract can be related to changes in concentration of these ions. We found that addition of the plant extract to the perfusion fluid did, however, lead to an increase in the magnesium- and potassium concentration of the experimental perfusion fluid (Table 2). The effect of increased $[\text{K}^+]$ as well as the effect of the combined increase in $[\text{K}^+]$ and $[\text{Mg}^{2+}]$ was thus investigated due to the observed increase in these elements with increased [*L. leonurus*] in the perfusion fluid.

Elevated $[\text{K}^+]$ of the perfusion fluid in the order of that observed in these experiments (to ca. 11 mM) results in loss of resting membrane potential and shortening of the action potential duration in isolated porcine heart paced with a basic cycle length of 430 to 500 msec (Morena et al. 1980). Adding KCl to the perfusion fluid is an acceptable way of increasing the $[\text{K}^+]$ of the perfusion solution and was also used by

Morena et al. (1980) who found that ventricular fibrillation occurred when the $[K^+]$ was increased to around 15 mM. Electrocardiographic recordings were not taken in the current study and we cannot comment on the electrophysiology.

$[Mg^{2+}]$ can influence cardiac contractility and rate (Specter et al. 1975). Hall and Fry (1992) reports that an increase of extracellular Mg^{2+} ($[Mg]_o$) reduced myocardial excitability and conduction. They also reported that raised $[Mg]_o$ was negatively inotropic which corresponds well with the current observations

that the LVEDP, LVESP, LVDP, HR, CW, and Q were reduced in the experimental solution containing Mg^{2+} (HighMgK). Although this increase in the $[Mg^{2+}]$ of the perfusion solution containing *L. leonurus* could thus have contributed to the decreased heart rate, the magnitude of the bradycardia as well as the magnitude of the negative inotropic response observed with LI was significantly greater than that observed during the HighMgK perfusion indicating the presence of other factors contributing to this phenomenon.

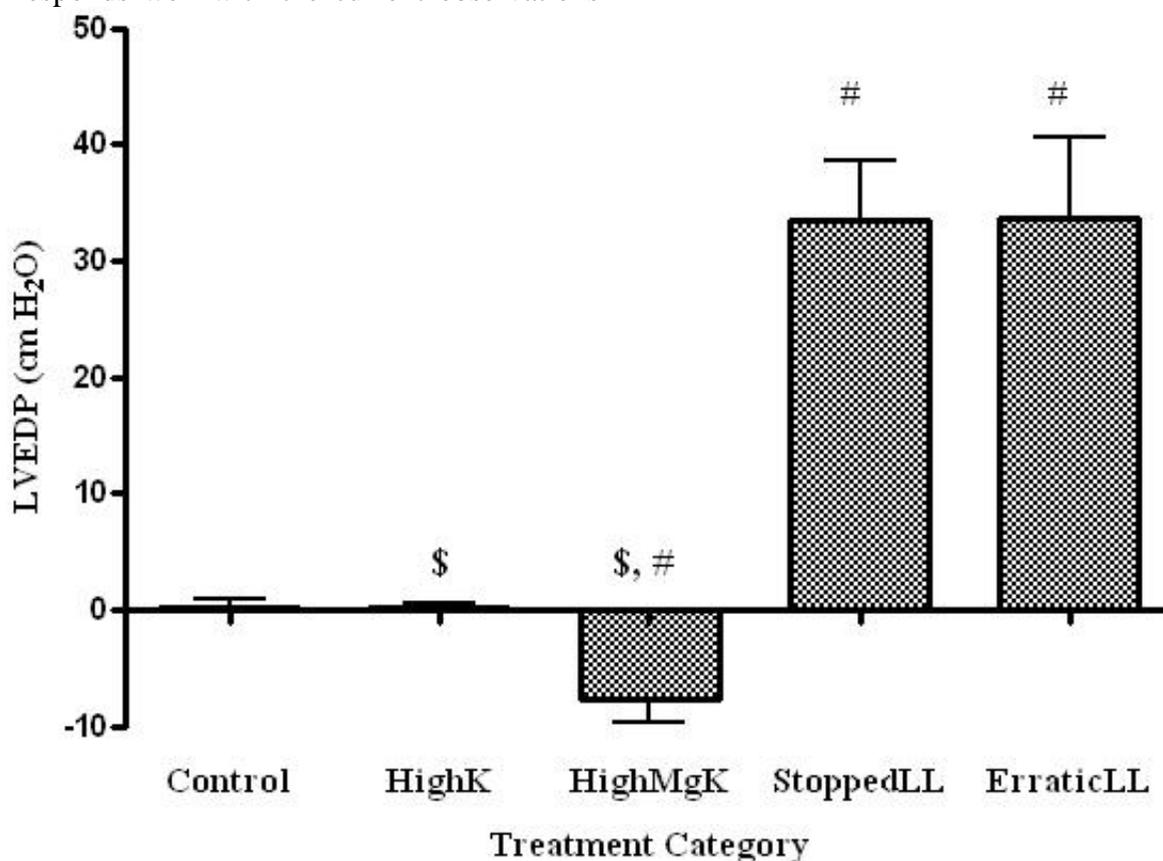


Figure 2: Change in Left Ventricular End Diastolic Pressure (LVEDP) (Mean \pm SEM) Control: Pure perfusion buffer (K-H); HighK: K-H with $[K^+] =$ K-H with 2 mg/ml *L. leonurus*; HighKMg: K-H with $[K^+]$ and $[Mg^{2+}] =$ K-H with 2 mg/ml *L. leonurus*; StoppedLL: Hearts that stopped with K-H containing 2 mg/ml *L. leonurus*. ErraticLL: Hearts that became erratic with K-H containing 2 mg/ml *L. leonurus*. #: differs significantly ($P < 0.05$) from control; \$: differs significantly ($P < 0.05$) from LI.

The K-H that formed the control perfusion fluid and the basis of the three test solutions was similar to K-H perfusion buffers that were used previously for perfusion of isolated rat hearts (Lochner et al. 2003; Lochner et al. 2006; Sonne et al. 2008). The control perfusion fluid in the current tests had a $[Ca^{2+}]$ of 1.3mM instead of the 2.5mM originally used by Krebs and Henseleit (1932). This $[Ca^{2+}]$ was used because

the actual ionized calcium concentration in plasma is approximately half that recommended by Krebs and Henseleit due to calcium binding to proteins and current investigators are using calcium concentrations in the range of 1.2-1.8 mM to prevent the hearts from working near the upper limits of the calcium/function curve (Sutherland and Hearse, 2000).

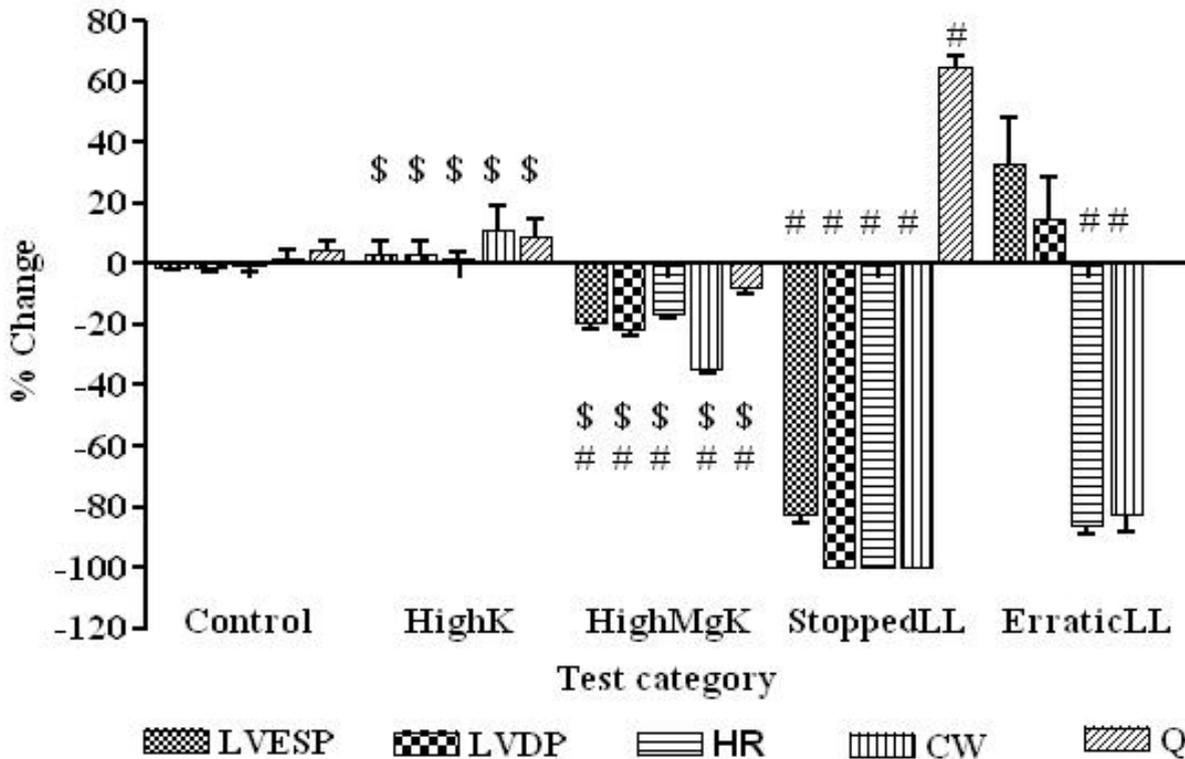


Figure 3: % Change in contractile parameters (Mean \pm SEM) Control: Pure perfusion buffer (K-H); HighK: K-H with $[K^+] = K-H$ with 2 mg/ml *L. leonurus*; HighMgK: K-H with $[K^+]$ and $[Mg^{2+}] = K-H$ with 2 mg/ml *L. leonurus*; StoppedLL: Hearts that stopped with K-H containing 2 mg/ml *L. leonurus*. ErraticLL: Hearts that became erratic with K-H containing 2 mg/ml *L. leonurus*. LVESP: Left ventricular end systolic pressure, LVDP: Left ventricular developed pressure; HR: Heart rate; CW: Cardiac work; Q: coronary perfusion #: differs significantly ($P < 0.05$) from control; \$: differs significantly ($P < 0.05$) from L1

Effect of 2 mg/ml *L. leonurus*

Some hearts stopped completely after 5 min perfusion with LI and this group was designated StoppedLL. In other hearts (designated ErraticLL) the HR was significantly reduced with the hearts giving spurious and irregular contractions after 5 min perfusion with LI. The effect of the LI administration is discussed under the specific headings in the following sections.

Left Ventricular End Diastolic Pressure (LVEDP)

Control diastolic pressure was 0.14 ± 2.13 cm H₂O. 2 mg/ml *L. leonurus* significantly increased diastolic pressure to 33.57 ± 12.31 cm H₂O in the hearts that stopped completely after 5 min exposure (StoppedLL) and to 33.66 ± 15.48 cm H₂O, in the erratic hearts (ErraticLL) (Figure 2). When expressed as a

percentage of pretreatment values the mean LVEDP in the ErraticLL hearts changed non-significantly by $686.92 \pm 1053.45\%$. Neither HighK (0.15 ± 0.98 cm H₂O) nor HighMgK (-7.77 ± 4.57 cm H₂O) influenced LVEDP to the same extent as 2 mg/ml *L. leonurus*. The change in LVEDP observed with the HighK solution was not significant relative to the control but differed significantly from that observed with LI. The decrease in LVEDP resulting from the HighMgK was significantly different from the control as well as from the LI effect.

Intraventricular pressure drops essentially to zero during ventricular diastole. One of the exclusion criteria was indeed that the diastolic pressure had to be below 13.6 cm H₂O (10 mm Hg) at the onset of the equilibration period. This is necessary in order to prevent over inflation of the ventricle. The PVC balloon in the left ventricle was therefore filled at the onset of the

experiment with distilled water to a volume that did not increase the diastolic pressure to more than 10 mmHg. Any small change in diastolic pressure could thus appear significant because the diastolic pressure started from a very low base, since a 1 mm Hg difference from a control reading of 1mm Hg would translate to a 100% change and a 1 mmHg difference from a control reading of 0.1 mm Hg would translate to a 1000% change. A similar 1 mmHg change in a developed pressure of 100 mmHg would however only lead to a 1% change. The actual change in diastolic pressure was therefore reported and not the % change as for the other parameters.

Expressing diastolic pressure after LI as a % change from the control situation gave ludicrous results ($686.92 \pm 1053.45\%$ increase) due to division by the very low changes in the control value. This means that this enormous percentage increase in LVEDP did not test significantly different relative to the pure K-H (control). Comparing the actual values of the diastolic pressures in cm H₂O after LI exposure with the control values however shows a significant increase in LVEDP after LI exposure. The increase in LVEDP observed with constant pressure perfusion after 5 min of LI exposure was not mimicked by either the HighK or the HighMgK experimental hearts. The reason for this increased LVEDP can therefore not be traced to the changes in inorganic ion concentration of the perfusion fluid.

Left Ventricular End Systolic Pressure (LVESP)

Control LVESP was 197.18 ± 24.84 cm H₂O. Control LVESP (Figure 3) changed by $-1.18 \pm 2.32\%$ when switched between the two hydraulic channels. The mean change in LVESP after HighK treatment ($3.23 \pm 10.41\%$) did not differ from the control. HighMgK ($-19.30 \pm 4.74\%$) and StoppedLI (-82.40 ± 6.65) decreased LVESP significantly relative to the control.

In ErraticLL, the mean LVESP increased by $33.01 \pm 33.83\%$, which was not significant. Actual LVESP after LI (69.10 ± 72.40 cm H₂O) did not change (unpaired t-test with Welch's

correction due to variances differing significantly) in ErraticLL hearts.

In the StoppedLL hearts, the HR (Figure 3) decreased significantly by $100.00 \pm 0.00\%$, while the LVEDP after 5 minutes of LI perfusion was increased (Figure 3). The LVESP cannot drop below the LVEDP and consequently it did not drop to zero but to LVEDP. The % change in LVESP was therefore not 100%, although the hearts stopped completely. In the ErraticLL hearts, the LVESP appeared increased but this was not significant. The large variability in this parameter accounted for this.

Left Ventricular Developed Pressure (LVDP)

Control LVDP was 202.80 ± 35.90 cm H₂O. LVDP (Figure 3) was significantly reduced by LI ($100.00 \pm 0.00\%$) in the hearts that stopped completely while the mean LVDP was increased by $14.59 \pm 31.38\%$ in the spuriously contracting hearts relative to control ($-1.17 \pm 2.85\%$). Actual LVDP ($+35.44 \pm 69.90$ cm H₂O) did not change significantly in ErraticLL hearts. Perfusion with the HighMgK solution in contrast caused a significant decrease in LVDP ($-21.65 \pm 4.22\%$) relative to control. The HighK did not change this parameter ($3.00 \pm 10.88\%$) significantly relative to the control. On the other hand, the LVDP obtained with LI perfusion differed significantly from that obtained with HighMgK as well as from that observed with HighK.

LI caused a significant decrease in the LVDP in the hearts that stopped but not in the hearts that showed a reduced but erratic heart rate ($-35.40 \pm 70.00\%$) while the HighMgK led to a significantly smaller decrease in the LVDP and the HighK group did not show any significant difference with the control (Figure 3).

Khan (2007) found an increase in LVDP while we report a significant decrease in the StoppedLL group and a non-significant increase in the ErraticLL group. This discrepancy may be due to the difference in the perfusion technique (constant pressure for 5 minutes in the current experiments vs. constant flow for 3 minutes). The relatively high SD in LVDP in ErraticLL should be noted. This accounted for the large mean % increase in LVDP in this group testing not significant.

Heart Rate (HR)

Control Heart rate was 264.86 ± 14.53 b.p.m. Mean heart rate was significantly reduced by the LI in both StoppedLL ($-100.00 \pm 0.00\%$) and ErraticLL $-85.97 \pm 6.44\%$. The actual HR decreased significantly by 229.60 ± 16.21 b.p.m. in the ErraticLL hearts. The HighMgK significantly reduced the HR ($-16.44 \pm 4.39\%$) while the HighK ($1.78 \pm 5.43\%$) did not change HR significantly relative to the control values ($-0.31 \pm 5.99(6)\%$). The % decrease in heart rate seen after five minutes LI exposure was significantly more than that resulting from either HighK or HighMgK exposure.

HR decreased significantly in StoppedLL and ErraticLL with only spurious contractions occurring in the latter group after 5 minutes perfusion with LI. In the erratic hearts, the actual changes in LVEDP, HR and CW differed relative to control.

Although the HighMgK also reduced the heart rate significantly, this decrease was much less and differed significantly from the bradycardia resulting from the LI exposure. Neither the HighK nor the HighMgK could thus explain the dramatic reduction in heart rate observed with 5 minutes constant pressure exposure to LI. Elevated KCl (to 16-17 mM) in K-H was used in another study to arrest hearts (Kupriyanov et al. 1995). The $[K^+]_o$ (extracellular $[K^+]$) in the current experiment (11 mM) was not increased to such high levels but it is noticeable that the HR was not significantly reduced by the HighK solution at 11mM K^+ . This is in agreement with the fact that sinus node activity still persisted when the sinus node was perfused with 10.8 mM potassium solution in anaesthetized open chest dogs (Vassalle et al. 1973).

Cardiac Work (CW)

The mean cardiac work (CW) under control conditions was 54455.05 ± 2988.53 cm H_2O .b.p.m. (40040.48 ± 2197.45 mmHg. b.p.m.). CW decreased significantly by $100.00 \pm 0.00\%$ relative to control in the StoppedLL hearts and by $82.91 \pm 11.52\%$ in ErraticLL hearts (Figure 3). CW also decreased significantly ($-34.56 \pm 4.35\%$) in the hearts treated with HighMgK. There was no significant difference in CW in

the HighK group ($11.00 \pm 19.48\%$) relative to control ($1.44 \pm 8.18\%$). The CW observed with the LI was significantly lower than that observed with both the HighK and the HighMgK perfusion solutions in the StoppedLL group.

The magnitude of the decrease in CW ($-100.00 \pm 0.00\%$ for the group where the hearts stopped completely and $-82.91 \pm 11.52\%$ for the erratic hearts) in the LI group is much larger than that observed in the experiments by Khan (2007) who observed a $48.84 \pm 4.89\%$ decrease in cardiac work after 3 minutes constant flow exposure to *L. leonurus*. This may be related to the longer exposure time in the current experiments and/or the difference in the perfusion mode.

Coronary Perfusion Rate (Q)

The mean coronary flow rate (Q) with the pure perfusion fluid was 13.40 ± 1.36 ml/min. Addition of 2 mg/ml *L. leonurus* to the perfusion fluid led to a significant increase in the coronary flow ($64.69 \pm 10.11\%$) (Figure 3) while the HighMgK ($-7.77 \pm 4.57\%$) solution caused a significant decrease in Q and the HighK ($8.66 \pm 14.16\%$) did not cause any significant change in this parameter relative to control (4.67 ± 6.45). Q after LI differed significantly from that after HighK as well as from that after HighMgK.

Hagen (1839) and Poiseuille (1846) did important work on the factors that govern the volume of flow through rigid tubes of capillary diameter *in vitro*. Based on this work, the Poiseuille-Hagen equation was later derived to describe the volume flow per unit time (Q): $Q = ((P_1 - P_2) r^4) / (8L \eta)$ where: $(P_1 - P_2)$: pressure head; η : viscosity ('a lack of slipperiness of the fluid') (Keele et al. 1982). In the intact constant pressure perfused heart the only variable in this equation is the inside radius of the tube. The inside radius of the blood vessels will therefore be the major variable that can change the resistance to flow.

The increased coronary perfusion rate (Q) observed on LI administration in this constant pressure perfusion model is in agreement with

the decreased perfusion pressure found by Khan (2007) in the constant flow perfusion system. The decreased perfusion pressure coupled to the increased $[K^+]$ in the LI served as further motivation for the current study because potassium has been implicated in vasodilation of coronary vasculature (Dawes 1941; Driscoll and Berne 1957). The reduced perfusion pressure observed by Khan (2007) in the constant flow model could have resulted from a primary decrease in the resistance in the coronary blood vessels as a result of changes in the blood vessel diameter (possibly mediated by an increase in the $[K^+]$) or as a secondary effect due to decreased external compression of the vessels caused by decreased contractility as discussed above.

Hiramatsu et al. (1998) stated that the intramyocardial vascular diameter changes which occur between systole and diastole are due to intramyocardial pressure and compressive forces resulting from contraction of myocytes. Gregg and Green (1940) also describe changes in coronary flow during the cardiac cycle. The increase in coronary flow observed in these constant pressure experiments in the current study could thus have resulted from decreased external compression due to reduced contractility of the ventricular musculature or from decreased vascular resistance due to blood vessel dilatation (possibly K^+ mediated). Although the observations with the constant flow and constant pressure techniques are therefore compatible, they cannot explain the underlying mechanism. The differences in the magnitude of the responses in the two studies may be related to the three minute perfusion period in the constant flow tests relative to the five minutes perfusion in these constant pressure experiments.

A critical comparison of the results obtained with the constant flow model (Khan 2007) and those reported here for the constant pressure model sheds more light on the primary cause for the decreased contractility. In the constant flow experiments (Khan 2007), it would be difficult to decide if the reduced contractility was the result of a primary decrease in the contractility of the left ventricular muscle or due to the reduced perfusion pressure observed with this constant flow technique. It should be noted that

a reduced coronary perfusion pressure in the constant flow experiments could lead to a decrease in contractility according to the Gregg effect (Gregg 1963). The results reported here, however, indicate that the reduction in LVDP was a primary effect and not a secondary effect due to the reduction in perfusion pressure because the LVDP was reduced in the current constant pressure experiment while the perfusion pressure did not decrease.

The increase in the coronary perfusion rate that was observed with LI in this constant pressure model could however have resulted from the decreased external compression caused by the decreased left ventricular contraction manifested in the decreased LVDP. This increased Q in the LI treated hearts was probably not due to the increased $[K^+]$ although the HighK group did show a non-significant increase in Q relative to control. The decreased Q in the HighMgK group may have an autoregulatory basis since the CW decreased significantly relative to control in this group. The increased Q in the StoppedLI hearts may be due to decreased external compression resulting from decreased cardiac contractility because the LVDP decreased significantly on LI administration.

The Perfusion model

Although the Langendorff perfused heart has been widely used in cardiovascular research, we modified the original technique as described in 1897 (Langendorff 1897).

Two main approaches are traditionally followed in Langendorff perfused heart models: a) the constant flow perfusion model where a constant volume is perfused through the coronary vasculature per minute and b) the constant pressure perfusion model where the perfusion pressure is kept constant (Sutherland and Hearse 2000).

In the constant flow perfusion model a specific constant volume of fluid is forced through the coronary circulation in a given time period (*e.g.* 8-12 ml min⁻¹ g⁻¹ wet wt. of tissue) by a constant volume pump system. To the contrary, in the constant pressure perfusion model, fluid is allowed to flow through the coronary circulation at a rate dependent on the

applied constant pressure (normally 60-100 mmHg) and the coronary vascular resistance (Sutherland and Hearse 2000). In the constant pressure model the heart is allowed to adjust the perfusion flow rate by automatic adjustments of the calibre of the vascular blood vessels and thus the coronary vascular resistance.

In the constant flow non-recirculating model, the injection of a known concentration of a test substance at a specific rate into the aortic cannula will result in exposing the heart to a specific concentration of the test substance for the duration of the injection plus the washout period afterwards. This can be seen as a non-physiological condition. However, in the constant pressure cardiac perfusion model (the model of choice for this investigation), the perfusion flow is determined by the autoregulatory mechanisms. Changes in coronary perfusion as was seen in this study (resulting from autoregulatory mechanisms or bioactive substances in the perfusion fluid) will however cause changes in the concentration of active substances to which the heart is exposed if the test substances are injected into the aortic cannula.

The above plus the general design of the study prompted us to use this modified double-sided Langendorff perfusion model where the test solution was not injected into the aortic cannula, but supplied from completely separate perfusion lines with their own reservoirs.

In addition to the concentration effects, *vide infra*, a further factor that influenced the decision to use this modified Langendorff system was the possibility that active substances can adsorb to the perfusion lines and be released later to influence the recovery or control periods. One side of the double sided perfusion system was always used for the active substances and the other side for the control substances. Hydraulic channels were switched with control perfusion fluid in both channels before experiments to check for possible systematic errors. None were observed.

Sutherland and Hearse (2000) came to the conclusion that “the isolated perfused small mammalian heart probably represents the optimal compromise in the conflict between quantity and quality of data that can be acquired

from an experimental model *vs.* its clinical relevance”. The double sided Langendorff perfusion system (Figure 1) where the control perfusion fluid was pumped through one channel and the test fluid was pumped through the other hydraulic channel that was used in this constant pressure perfusion system would thus present decided advantages to a perfusion system where a test substance is simply injected into the aortic cannula.

The use of the constant pressure model in this study also shed more light on the relationship between the reduced contractility, the perfusion pressure and Q.

The relationship between contractile strength and coronary perfusion

Contraction of the heart muscle interferes mechanically with the blood flow through the blood vessels in the heart wall. In the left ventricle of the dog at rest, there is a complete stop in the coronary blood flow during the isometric contraction and a backflow is actually established, but a peak coronary flow is established when the systolic pressure is at a maximum during the early ejection phase of the cardiac cycle. A second peak flow is established during early diastole, after which flow drops to about 70% of maximum just at the end of diastole (Gregg and Green 1940). Cardiac contractility therefore influences the vascular resistance because the cardiac blood vessels may be compressed if the increased pressure outside these vessels that is generated by the contraction of the myocardium exceeds the perfusion pressure inside these blood vessels. Thus, in the constant flow model, increased perfusion pressure will be seen during ventricular systole due to the increased vascular resistance caused by the compression of the blood vessels. Uncontrolled changes in the perfusion pressure are not desirable because it will cause changes in the exchange of active substances between the perfusion fluid and the active tissue.

Autoregulation of local blood flow ensures that tissues in general (with some notable exceptions being the kidneys and the liver) receive a specific volume of blood per minute that is proportional to the metabolic activity of

that specific tissue at the time. If a specific substance causes an increase in cardiac contractility, it will consequently increase the metabolic demand and result in decreased vascular resistance and increased coronary flow at a constant aortic pressure. This increase in coronary perfusion would thus be a secondary effect resulting from the increased metabolic demand. If a test substance however causes decreased vascular resistance (e.g. by directly causing an increase in vascular calibre) it may also cause an increase in tissue perfusion at a constant aortic pressure which would be a primary effect. Decreased metabolism and increased vascular resistance can similarly cause a secondary and a primary decrease in coronary perfusion. In both hypothetical scenarios, the flow rate of the perfusion fluid through the coronary circulation will be changed.

If the rate of infusion of the test substance is kept constant while the coronary flow rate changes, the concentration of this active substance to which the heart is exposed will change which will make the interpretation of the results difficult. If it is assumed that there is no significant metabolic breakdown or modification of the test substance in the *in vitro* system, the concentration of the test substance to which the heart is exposed is dependent on the flow rate of the perfusion fluid, the flow rate of the infused test solution containing the active substance and the concentration of the active substance(s) in the infusion solution.

Conclusion

It should be noted that both this study and the study by Khan (2007) used isolated perfused rat hearts whereas Scott and Springfield (1999) report that *Leonotis leonurus* Herba (the dried aerial part of the plant) is used mainly in the form of an aqueous decoction, orally, per rectum and as a topical application. Thus, the results may not be directly extrapolated to the traditional use of the plant, but the information is very valuable as to the possible biological action.

HighK had no significant effect on any of the parameters and although the HighMgK could have contributed to the decrease in LVESP, LVDP, HR, and CW following LI

exposure, the response to LI was significantly larger than that of HighMgK. Neither the increased $[K^+]$ in the perfusion solution containing 2 mg/ml *Leonotis leonurus* extract, nor the combined increased $[K^+]$ and $[Mg^{2+}]$ ions can therefore explain the observed changes in the cardiac parameters that resulted from the addition of the *L. leonurus* extract to the perfusion fluid. The reason for the changes in the contractile parameters of the constant pressure perfused isolated rat heart must be found in something other than either the increase in $[K^+]$ alone or the combined increase in $[K^+]$ and $[Mg^{2+}]$ in the perfusion fluid containing *L. leonurus* (2 mg/ml) since increasing these parameters to levels found in the LI did not lead to changes in the cardiac parameters of the same magnitude as that seen with LI. It can therefore be unequivocally stated that "The altered ionic composition of the perfusion fluid resulting from the addition of a *L. leonurus* plant extract (2mg/ml) to the perfusion buffer does not explain the changes in contractility observed in the isolated perfused Wistar rat heart". It follows that there must be chemical(s) other than the inorganic salts present in the *L. leonurus* extract that cause the observed effects on the retrograde isolated Langendorff-perfused rat heart.

This study also illustrates that one must be cautious when perfusing hearts with aqueous extracts of plant materials in K-H for the investigation of cardio-active effects since simple ionic changes in the perfusion fluid composition may occur and confound results.

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Abbreviations used: **K-H:** Krebs-Henseleit perfusion buffer, **LVEDP:** Left ventricular end diastolic pressure is the lowest pressure observed in the cardiac cycle, **LVESP:** Left ventricular end systolic pressure is the highest pressure observed in the cardiac cycle, **LVDP:** Left ventricular end developed pressure is the difference between LVSP and LVEDP, **HR:** Heart rate is the number of heart beats per minute, **Q:** The coronary perfusion rate is the volume of effluent (ml) from the isolated perfused heart per minute, **CW:** The cardiac work is the product of the HR and the Q, **L1:** 2 mg/ml *Leonotis leonurus* aqueous extract in Krebs-Henseleit buffer, **HighK:** Krebs-Henseleit buffer to which KCl was added to give a buffer solution with a potassium concentration of 11 mM, **HighMgK:** Krebs-Henseleit buffer to which KCl and MgCl₂ was added to give a buffer solution with a K⁺-concentration of 11 mM and a Mg²⁺-concentration of 17mM, **StoppedLL:** Those hearts that stopped beating after they were exposed to 5 min perfusion with L1 (n=6), **ErraticLL:** Those hearts that did not stop beating after they were exposed to 5 min perfusion with L1 but only showed a marked reduction in the heart rate and the cardiac work with an increase in contractility (n=5)
