Hypoglycemic effect of oral administration of the infusion of *Leptocarpha rivularis* in alloxan- induced diabetic rats.

Carolina Álvarez-Contreras[1], Rolando Martínez[1], Héctor Carrasco-Altamirano[1], María Cristina Chamy[1], Claudia López[2], Víctor Cubillos[2], Gloria León[3], Horst Grothusen[3], Hernán Palma-Fleming[4], Iván Montenegro[5], Luis Espinoza[5], Enrique Werner[6]

[1]Departamento de Química. Facultad de Ecología y Recursos Naturales. Universidad Andrés Bello. Los Fresnos 52. Viña del Mar. Chile. *Email* rmartinez@unab.cl.
[2]Instituto de Patología animal. Facultad de Ciencias Veterinarias. Universidad Austral de Chile. Valdivia. Chile.
[3]Instituto de Bioquímica. Facultad de Ciencias. Universidad Austral de Chile. Valdivia. Chile.
[4]Instituto de Química. Facultad de Ciencias. Universidad Austral de Chile. Valdivia. Chile.
[5]Universidad Técnica Federico Santa María, Av. España 1680, Valparaíso, Chile.
[6]Departamento De Ciencias Básicas, Universidad del Bio-Bio. Chillán. Chile.

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Abstract

Type II diabetes mellitus (NIDDM) is a condition in the body when the pancreas does not produce enough insulin to process glucose, or insulin receptors are not functioning properly. Today more people are diagnosed with NIDDM due to an increasing obesity and sedentary lifestyle. In many places around the world, diabetes is kept under control with medicinal plants used for primary health care. This study investigates the hypoglycaemic effects of oral administration of the infusion of *Leptocarpha rivularis* in diabetic rats induced by alloxan. The treatment of diabetic rats with infusion not only showed significant anti-hyperglycaemic effect but also lipid-lowering effect. The results were compared between normal and diabetic rats with and without treatment. Considering the favourable results, it is necessary to evaluate the use of this infusion as an alternative treatment for diabetes.

Introduction

Diabetes mellitus remains as a major public health problem in the world since the number of people suffering from this disease increases every day. This epidemic is caused mainly by genetic and environmental factors such as obesity and lack of exercise. It is a growing problem in children and young adults, which gives a devastating point of view to think about the age of onset of the disease. Diabetes mellitus is the most important chronic disease involving the endocrine pancreas. It is characterized by hyperglycemia and biochemical abnormalities in the metabolism of glucose and lipids due to defects in insulin secretion, insulin action or both. The liver is a tissue-dependent insulin that plays a key role in the homeostasis of glucose and lipids, and is severely affected during diabetes (1). Nowadays there is a variety of oral hypoglycemic agents (OHA); however, almost all of them show unwanted effects on the quality of life of patients with diabetes (2-3). As mentioned above, many research groups in the world have devoted their efforts to find new therapeutic options. Many plants used in traditional medicine for the treatment of diabetes mellitus have been used throughout the world and only some of them have been subjected to scientific evidence (4-5). According to the World Health Organization this area deserves attention.

This paper describes the study of *Leptocarpha rivularis* D.C-, a member of the tribe Heliantheae (Compositae)- which has been used as a popular medicine in the Mapuche culture in southern Chile (6). As part of our research on secondary metabolites of compositae with potencial biological activity, an earlier examination of the plant has yielded as the principal component leptocarpin (7). Also, 17,18-dihydroleptocarpin and rivularin (8) were found. Structural and conformational studies of the compounds were based on elemental analysis (IR spectroscopy, ^{13}C-NMR and ^{1}H-NMR experiments). All these compounds are germacranolide, and belong to the heliangolide subgroup. Many of this type of the sesquiterpene lactones have shown to possess diverse biological activity. **Figure 1.**

The purpose of this study is to evaluate the effect of the infusion on the fasting serum glucose, total cholesterol and triglycerides levels during a ten week treatment. To this end, healthy and diabetic rats induced by alloxan were considered. At the same time, a chemical analysis using GC-MS of the composition of the infusion was made to possibly find the compounds responsible for the observed activity.

Materials and Methods

Chemicals

Alloxan was obtained from Sigma Aldrich. All other reagents used during this research were of analytical grade.

Preparation of plant extract

The plant used was collected during a three month period (January to March 2003) in the surroundings of Valdivia city (Chile). The plant was identified and classified by qualified people, and a voucher specimen representing this collection was deposited at Herbarium of Botanical Institute (Vald. 13006), Universidad Austral de Chile.

The infusion dose was selected according to three different treatments with 22 mg, 36 mg and 60 mg (single daily dose for 15 days) in the pre-experimental phase. A concentration of 36 mg L.rivularis infusion was chosen to represent a best antidiabetic effect in the animal treatment.
According to the above-mentioned, an amount of 4.5 g (leaves and branches) was mixed with 250 ml of water and then boiled for 10 minutes. The mixture was cooled at room temperature, filtered through fluted filter paper, and maintained at 4 ^{0}C until its administration. A volume of 2.0 ml of the aqueous solution was given daily to each rat using an esophagic tube.

Chromatographic analysis of the extract
The analysis of the 2µL sample composition of the *Leptocarpha rivularis* infusion was conducted in a Hewlett Packard 6890gas chromatograph coupled to a Hewlett Packard 5973 mass selective detector and equipped with a capillary column HP- 5MS 5% Phenyl Methyl Siloxane column HP 19091S – 433, capillary 30.0 m x 250 µm x 0.25 µm and a system for NIST data analysis. The initial program temperature was 275ºC (splitless injection) with a temperature ramp of 40ºC/min up to 250ºC. Flow rate of He was 1.3 ml min^{-1} at 10.12 psi was applied. GC data are reported with a retention time and % area of the total integrated area.
The compound identification in the chromatographic profiles was achieved (Figure 2) by comparing their mass spectra with a library data base. Spectra were considered coincident if the similarity index was higher than 95%. Preliminary identifications were confirmed by observing peak enhancements upon standard co-injection: 8β-Angeloyl-1β,3β- dihidroxy-4,10-dimethyl-$\Delta^{11(13)}$ methylen-4Z,9Z-dienheliangol- 6,12-olide and 8β-Angeloyl-1β,3β-dihidroxy-4-methyl-$\Delta^{11(13)}\Delta^{10(14)}$-dimethylen-4Z-enheliangol-6,12-olide obtained according to the methodology described in reference (9).

Animals and experimental set-up
All the experiments were carried out with male Sprague-Dawley rats (350-400 g) obtained from “Bioterio Central de la Facultad de Ciencias Médicas”, Universidad de Chile. The animals were fed *ad libitum* with standard laboratory diet and water. Animals were maintained under a constant 12 h light and dark cycle and at a room temperature of 22-24 ^{0}C. The animals used in this study were maintained according to the guidelines for research animals at Universidad Austral de Chile.
Diabetes induction was accomplished by three alloxan intraperitoneal injections (Sigma-Aldrich, USA) at 48 hour intervals. Alloxan dosage supplied was of 75 mg/Kg body weight prepared in saline solution. Four weeks after the first injection, the rats were fasted and blood glucose levels were recorded. The blood samples were taken- in tubes containing EDTA- from the orbital sinus with the help of a capillary tube of rats under anaesthesia (diethyl ether). To minimize the effects of daily fluctuations, blood samples were collected at the same hour in every experimental time.
Animals were considered to be diabetic when plasma glucose concentrations were higher than 200 mg/dl. The totality of rats under study, to which diabetes was induced, developed the clinical signs of the disease (polyuria, polydipsia, polyphagia and loss weight). After this, the animals were divided into four

experiment groups of nine rats each one, as follows: Control rats; Control + infusion rats; Diabetic rats and Diabetic + infusion rats.
After a 30 day period of diabetes induction, the animals were deprived of food overnight. Blood was collected before starting the treatment in every experimental time; all the samples were stored at -20 ^{0}C until analyzed. The following experimental times were considered: Time 0: Before starting the treatment; Time 1: four weeks after starting the treatment; Time 2: seven weeks after starting the treatment; Time 3: ten weeks after starting the treatment.

Analytical procedure

The chosen methods are those traditionally used in laboratories of clinical chemistry by enzymatic colorimetric methods using commercial kits (Valtek Diagnostics, Santiago, Chile), as detailed as follows: Fasting blood glucose was estimated by glucose oxidase method; concentrations of total cholesterol and triglycerides were estimated by the cholesterol and lipase oxidase method; the amounts of protein and plasmatic albumin were determined by the Biuret and Bromocresol green methods.

Determination of thiobarbituric acid reactive substances in plasma

Lipid peroxides (TBAS) in plasma were estimated using thiobarbituric acid reactive substances by the Asakawa and Matsushita method (1979), modified by Estepa (10). Briefly, to 100 µl of plasma, 100 µl antioxidant react (2.2 g/L BHT in ethanol), 100 µL catalyzing react (2.7 g/L of ferric trichlroride in water) and 1.5 mL of buffer (75.05 g of glycocoll and 58.44 g of NaCl per 1 liter at pH = 3.5) were added. The mixture was incubated at 5^{0}C for 60 min and after heated in a water bath at 95^{0}C for 60 min using a glass ball as condenser. 2.5 mL of n-butanol/pyridine mixture and 0.5 mL of water were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was measured its absorbance at 532 nm. 1,1,3,3 – tetramethoxypropane (Sigma-Aldrich, USA) was used as standard. The level of lipid peroxides was expressed as µmol/L of TBA.

Results:

In the qualitative analysis for the *L. rivularis* infusion by GS-MS, the following composition was identified as detailed in the table 1. Its analysis showed that monoterpenoids and germacrene D are present in the infusion; it is not an unusual finding because they are precursors of the SLs biosynthetic pathway. These compounds were present in high concentrations; specifically, the heliangolidas type together with many other metabolites with antioxidant activity recognized as the phenolic compounds Phenol, 2,4-bis (1,1-dimethylethyl) and Phenol, (1,1,3,3-tetramethylbuthyl). (Figure 1).

Four weeks after diabetes induction, significant increases in the blood glucose level were observed in rats when compared to control rats within time cero. The infusion supply to diabetic rats (Diabetic + infusion group) decreased the blood glucose level and values similar to the observed in normal rats were found

(Table 2). On the other hand, the blood glucose values in control + infusion rats did not display a significant decrease with regard to the control group.
We identified that *L. rivularis* not only showed an anti-hyperglycemic effect but also an hypolipidemic effect. Results showed the beneficial effect on the fasting plasma cholesterol and other biochemical parameters analyzed (TC, LDL, HDL y TAG) showed in Tables 3, 4, 5 y 6 respectively. A marked increase in the frequency of cholesterol and LDL fraction was observed in diabetic untreated rats, while the rats treated with the infusion significantly reduced the lipid levels after a 10 week of treatment.
The results shown in table 7 confirm that the lipid peroxide scavenging activity of *L. rivularis* administration also reduced lipid peroxidation. Very marked decreases in the lipoperoxids levels of both rat groups were dealt daily with plant infusions.
In the study, albumin concentration values and total proteins were analyzed as well. Statistics differences among the different experimental groups were not observed to the different considered times (table 8 and 9).

Discussion

In medicine and in other medicinal ancient systems of the world, many plants have been used for mellitus diabetes treatment. Perhaps, only a few have been evaluated by modern medicinal systems which generally have less confidence in the infusion treatments.
In our study, we have found that the *L. rivularis* infusion showed anti-hyperglycemic effect in alloxan diabetic rats without affecting healthy subjects. Like the hypolipidemic effect, it is interesting to note that hyperlipidemia is a common problem for the mellitus diabetes patientsthat increases the risk of associated cardiovascular diseases (11).
In the analysis of the albumin concentration values and total proteins, statistics differences between the different experimental groups were not observed to the different considered times. The reason why an antidiabetic effect considered as the cause Number 1 by the insulin administration is not possible . When this happens, the plasmatic protein concentration (mainly of albumin levels) increases, due to the anabolic insulin action on the metabolism (12-13).

For years SLs have been commonly isolated from numerous genera of the Compositae. A high percentage of the evaluated sesquiterpenes have showed activities such as antimicrobial, antiviral, antiangiogenesis, citotoxicity. Furthermore, SLs were found to have anti-inflammatory properties (14-15), and most recently in monoterpenoids (16-17). As the inflammatory processes are related to the modulation of the immune response in mammals, these compounds contribute to regulate biological mechanisms, key in the treatment of infectious diseases, such as diseases related to the immune system, oxidative stress, diabetes or cancer, among others (18).
Great part of these studies in SLs had shown that these activities are mainly attributed to the presence of α, β unsaturated carbonyl moiety. These groups are known to be able to act with biological nucleophiles, especially cysteine sulfhydryl groups by a selective alkylation through Michael-type addition, considered the primary targets of SLs (19-20). Within these types of reactions, it is possible to observe interactions such as covalent modification of proteins and

polypeptide chains, or also the capacity to yield hydrogen bond in the vicinity of the alkylating site, thus stabilizing the covalent binding. Examples of this type of reactions occur in vivo with enzymes of the lipid synthesis like acetyl CoA, acetylCoA synthetase, citrate lyase (21). This reason could explain the lowering effect of the observed serum lipids. Many of the mentioned studies demonstrate that the interaction of different remainders of cysteine is found generally in the activation loop of their catalytic domain, implied in the regulation of cellular processes (22-23).

It is also discussed as present differences in activity among individual SLs. This may be explained by numbers of alkylating structural elements, lypophilicity and molecular geometry, as well as by other characteristics in the different answers (24-25-26) provided. All previously displayed in pharmacological research seeks for a potential therapeutic use.

Nevertheless, the hypoglycemic effect of sesquiterpene lactones is poorly understood. Even findings are contradictory for such isolated compounds (27-28). When the results have been positive, possible mechanisms of action have been suggested as responsible for this effect (28). As well known, this sesquiterpenoid types alter the cellular processes responsible for the electron transport and oxidative phosphorylation (29). This would explain the lowering effect on the glucose level.

An important observation is the fact that inhibition of the oxidative phosphorylation produces an enhanced glucose uptake carried out by metabolic cells and tissues through the glycolytic pathway in order to maintain the normal ATP cell levels. With the objective to maintain the glucose availability in the cell, an increase of translocation of the transporting glucose proteins takes place (GLUTs). This represents a fundamental adaptation that is critical to the maintenance of cellular homeostasis (30-31-32).

When 2.4 dinitrophenol (DNP) was assayed in vitro (a chemical compound that uncouples the oxidative chain), it has been proven to cause the mobilization of GLUT 1 and GLUT 4 to the cellular membrane without the insulin used as a mediator (33). The greater expression in the membrane of proteins GLUTs produces a decrease in the circulating glucose levels, and an increase in the glucose tolerance. As a result, many studies are based on this to point out therapeutic alternatives and effective therapies for type 2 diabetes (34).

Moreover, the presence of monoterpenoids with antioxidant action in the infusion can contribute to avoid the formation of SLs-GHS adducts through competitive inhibition with SLs. Due to this, the presence of monoterpenoids could increase the SLs bioavailability under physiological conditions. The consumption of smaller doses are needed to obtain the awaited effects without forgetting the monoterpenoids properties by themselves. Under this point of view, the administration of the infusion seems to be more beneficial after studying the activity of its components in isolated form. As previously discussed by Wagner et al (35), it is viable to think about its possible use as a pharmaceutical therapy.

On the other hand, it is interesting to mention that monoterpenoids have displayed antiulcer activity without affecting the gastric secretion H^{+14}, which together with the anti-inflammatory action of some infusion components could avoid some of the well-known annoyances caused by traditional therapy OHA currently used.(36).

Conclusions

According to the previously exposed, the effectiveness of the results could determine that the *Leptocarpha rivularis* infusion is a natural antidiabetic agent which can be used to control glucose levels as well as high cholesterol. However, it becomes necessary to make numerous studies to evaluate the compounds responsible for the beneficial effect of *Leptocarpha rivularis* on the control of the diabetic state and to understand the responsible mechanism of antidiabetic action. Possible unwanted effects should be discarded because these were not observed in diabetic animals or in healthy animals to which the infusion was administered.

Acknowledgments

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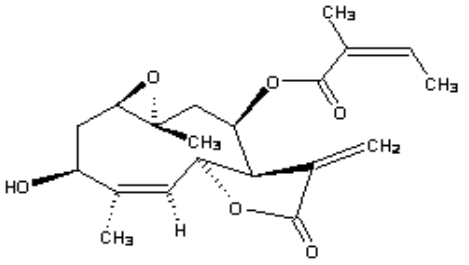
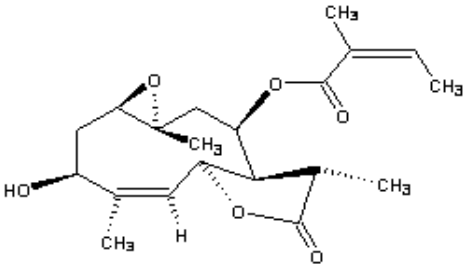
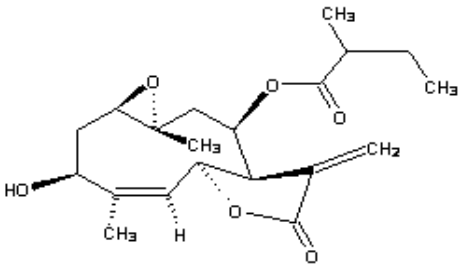
Figure 1.- Sesquiterpenes present at the aqueous infusion of Leptocarpha rivularis



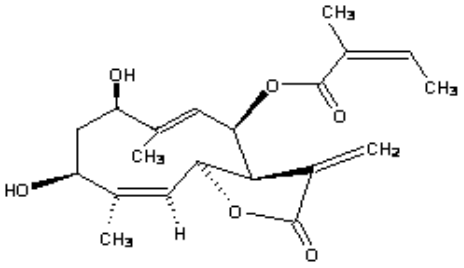
Leptocarpin



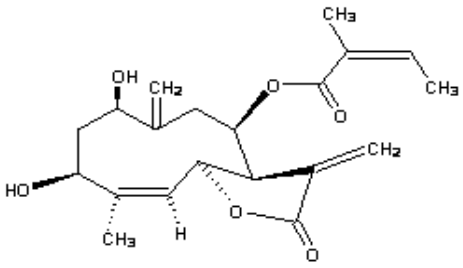
Rivularin



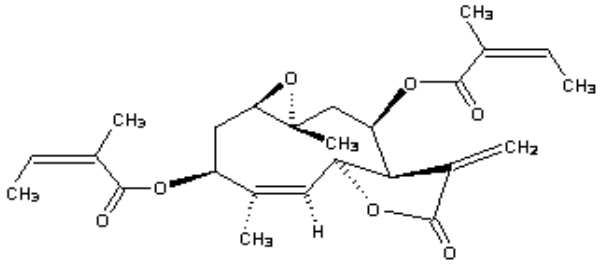
17,18-dihydroleptocarpin



8β-Angeloyl-1β,3β- dihidroxy-4,10-dimethyl -$\Delta^{11(13)}$ methylen-4Z,9Z-dienheliangol- 6,12-olide



8β-Angeloyl-1β,3β- dihidroxy-4-methyl-$\Delta^{11(13)}$ $\Delta^{10(14)}$-dimethylen-4Z-enheliangol-6,12-olide



3β-Angeloyl leptocarpin

Table 1.-GC-mass of infusion of *Leptocarpha rivularis*

Compound	**RT**	**Area** (%)
1.- Trans ocimene	4.24	3.08
2.- Bencene, 4-ethyl-1,2-dimethyl	4.92	0.07
3.- α-Thujone	5.19	2.25
4- D-β-Thujone	5.20	0.63
5.- α-Longipinene	8.34	2.09
6.- (-)-α-Copaene	8.61	0.23
7.- β-Caryophyllene	9.15	10.67
8.- α-Caryophyllene	9.55	0.55
9.-Germacrene D	9.85	4.55
10.-Phenol, 2,4-bis(1,1-dimethylethyl)-	10.76	0.26
11.- Phenol, (1,1,3,3-tetramethylbuthyl)	11.14	0.27
12.- Rivularin	12.11	3.50
13.- Octadecanoic acid, methyl ester	16.45	1.48
14.- 9-Octadecenoic acid, (E)-	16.57	0.45
15.- Octadecanoic acid	16.74	0.20

16.- n- Hexacosane	19.90	0.12
17.- 17,18- dihydroleptocarpin	19.93	4.92
18.- Tetracosane, 2,6,10,15,19,23-hexamethyl-	20.28	0.66
19.- 3β-Angeloyl leptocarpin	20.64	18.19
20.- Leptocarpin	21.01	46.18
21[a].- 8β-Angeloyl-1β,3β- dihidroxy-4,10-dimethyl-$\Delta^{11(13)}$ methylen-4Z,9Z-dienheliangol- 6,12-olide	21.33	2.55
22[a].- 8β-Angeloyl-1β,3β- dihidroxy-4-methyl-$\Delta^{11(13)}$ $\Delta^{10(14)}$-dimethylen-4Z-enheliangol- 6,12-olide	21.99	0.63

[a]Structure determined by direct comparison with authentic samples obtained according to the methodology described in reference 20.

Table 2. Effect of the treatment with infusion of *L. rivularis* on glucose levels(mg/dL) of normal and diabetic rats for each experimental time period. The values represent the mean ± S.E.M, by Dunnett's test.

Time	Control	Control + infusion	Diabetic	Diabetic + infusion
T0	145.1 ± 5.8	134.6 ± 4.0	256.3 ± 22.5[b]	284.6 ± 31.1[b]
T1	153.1 ± 6.3	152.9 ± 7.4	318.3 ± 25.3[b]	193.4 ± 12.9
T2	158.0 ± 4.4	142.0 ± 4.8	426.5 ± 49.6[b]	174.3 ± 3.6
T3	166.7 ± 22.7	110.7 ± 22.0	346.7 ± 76.6[a]	170.3 ± 26.2

[a] $p< 0.05$; [b] $p< 0.01$ v/s group Control .

Table 3. Effect of the treatment with infusion of *L. rivularis* on Cholesterol levels of normal and diabetic rats for each experimental time period. The values represent the mean ± S.E.M, by Dunnett's test.

Time	Control	Control + infusion	Diabetic	Diabetic + infusion
T0	94.0 ± 5.1	85.0 ± 4.5	94.4 ± 4.9	102.5 ± 2.7
T1	97.0 ± 4.7	93.5 ± 4.8	123.5 ± 9.8[a]	102.0 ± 3.4
T2	129.7 ± 3.8	125.5 ± 3.9	127.3 ± 2.7	118.3 ± 3.1
T3	142.9 ± 3.2	108.3 ± 1.7[a]	140.6 ± 12.4	111.0 ± 1.7[a]

[a] $p< 0.05$ v/s group Control

Table 4. Effect of the treatment with infusion of *L. rivularis* on HDL- Cholesterol levels (mg/dL) of normal and diabetic rats for each experimental time period. The values represent the mean ± S.E.M, by Dunnett's test.

Time	Control	Control + infusion	Diabetic	Diabetic + infusion
T0	57.1 ± 2.7	56.2 ± 1.6	55.2 ± 2.6	64.6 ± 3.4
T1	53.0 ± 0.7	63.7 ± 4.8	37.0 ± 2.9[b]	61.7 ± 4.4
T2	38.8 ± 1.1	46.0 ± 1.5[a]	40.3 ± 1.7	50.0 ± 2.1[a]
T3	32.0 ± 2.1	43.3 ± 1.8[a]	38.6 ± 0.8	46.7 ± 2.4[a]

[a] $p< 0.05$; [b] $p< 0.01$ v/s Control.

Table 5. Effect of the treatment with infusion of *L. rivularis* on LDL- Cholesterol levels (mg/dL) of normal and diabetic rats for each experimental time period. The values represent the mean ± S.E.M, by Dunnett's test.

Time	Control	Control + infusion	Diabetic	Diabetic + infusion
T0	13.8 ± 0.6	8.8 ± 0.5[b]	26.6 ± 0.5[b]	24.1 ± 0.7[b]
T1	17.5 ± 0.6	6.7 ± 0.2[b]	21.6 ± 0.6[b]	21.6 ± 0.5[b]
T2	52.4 ± 1.6	54.0 ± 3.9	68.8 ± 1.2[b]	37.2 ± 1.9[b]
T3	82.7 ± 1.8	45.7 ± 1.3[b]	87.5 ± 2.8	52.5 ± 1.4[b]

[b] p< 0.01 v/s group Control.

Table 6. Effect of the treatment with infusion of *L. rivularis* on Triglycerides levels (mg/dL) of normal and diabetic rats for each experimental time period. The values represent the mean ± S.E.M, by Dunnett's test.

Time	Control	Control + infusion	Diabetic	Diabetic + infusion
T0	111.2 ± 9.7	103.3 ± 8.5	57.8 ± 5.2[b]	68.2 ± 3.9[b]
T1	96.4 ± 8.8	117.4 ± 8.1	95.3 ± 8.4[b]	103.1 ± 11.2[b]
T2	161.0 ± 15.6	129.8 ± 6.4	88.2 ± 9.5[b]	118.2 ± 7.1
T3	148.7 ± 8.7	97.0 ± 13.0	51.0 ± 3.2[b]	85.0 ± 4.7[b]

[b] p< 0.01 v/s group Control.

Table 7. Effect of the treatment with infusion of *L. rivularis* on levels of lipoperoxides in serum levels (µmol/L) of normal and diabetic rats for each experimental time period. The values represent the mean ± S.E.M, by Dunnett's test.

Time	Control	Control + infusion	Diabetic	Diabetic + infusion
T0	2.1 ± 0.2	2.2 ± 0.5	2.5 ± 0.6	2.6 ± 0.2
T1	2.8 ± 0.2	2.2 ± 0.9	2.4 ± 0.9	2.0 ± 0.5
T2	1.6 ± 0.5	0.5 ± 0.1	3.5 ± 0.4[b]	0.18 ± 0.1[a]
T3	3.2 ± 1.0	0.7 ± 0.1	9.4 ± 0.7[b]	0.13 ± 0.1[a]

[a] p< 0.05; [b] p< 0.01 v/s group 1.

Table 8. Effect of the treatment with infusion of *L. rivularis* on total proteins (g/dL) of normal and diabetic rats for each experimental time period. The values represent the mean ± S.E.M, by Dunnett's test.

Time	Control	Control + infusion	Diabetic	Diabetic + infusion
T0	7.1 ± 0.5	7.3 ± 0.5	7.5 ± 1.5	7.4 ± 0.4
T1	7.3 ± 0.5	7.8 ± 0.3	7.0 ± 0.7	7.8 ± 0.7
T2	6.7 ± 0.4	7.2 ± 0.5	7.5 ± 0.5	7.0 ± 0.4
T3	7.6 ± 0.9	6.4 ± 0.7	8.4 ± 1.0	7.0 ± 0.4

Table 9. Effect of the treatment with infusion of *L. rivularis* on albumin levels (g/dL) of normal and diabetic rats for each experimental time period. The values represent the mean ± S.E.M, by Dunnett's test.

Time	Control	Control + infusion	Diabetic	Diabetic + infusion
T0	3.3 ± 0.2	3.1 ± 0.2	3.4 ± 0.1	3.3 ± 0.1
T1	3.3 ± 0.2	3.5 ± 0.3	3.4 ± 0.2	3.5 ± 0.6
T2	3.5 ± 0.4	3.7 ± 0.4	3.4 ± 0.2	3.2 ± 0.3
T3	3.7 ± 0.3	3.4 ± 0.2	3.7 ± 0.2	4.2 ± 0.1

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