



EDILENE BEGA FERREIRA

**AVALIAÇÃO DO POTENCIAL ANTIDIABÉTICO DA STEVIA
REBAUDIANA (BERT) BERTONI E DA AVERRHOA CARAMBOLA
L. (OXALIDACEAE) EM RATOS DA LINHAGEM WISTAR**

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Orientador: Dr. Roberto Barbosa Bazotte

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APRESENTAÇÃO DO TRABALHO

De acordo com as normas estabelecidas pela Coordenação do Programa de Pós-Graduação em Ciências Biológicas, esta tese de Doutorado foi redigida na forma de artigos científicos.

Edilene Bega Ferreira, Francisco de Assis Rocha Neves, Marlom Anselmo Duarte da Costa, Wilson Alves do Prado, Letícia de Araújo Funari Ferri, Roberto Barbosa Bazotte. Comparative effects of *Stevia rebaudiana* (Bert.) Bertoni leaves and stevioside on glycaemia and hepatic gluconeogenesis. **Planta Medica.**

Edilene Bega Ferreira, Luiz Cláudio Fernandes, Sharize B. Galende, Diógenes A.G. Cortez, Roberto B. Bazotte. Hypoglycemic effect of the hydroalcoholic extract of leaves of *Averrhoa carambola* L. (Oxalidaceae). **Phytoterapy Research.**

COMPARATIVE EFFECTS OF STEVIA REBAUDIANA (BERT.) BERTONI LEAVES AND STEVIOSIDE ON GLYCAEMIA AND HEPATIC GLUCONEOGENESIS.

ABSTRACT

The purpose of the present study was to compare the effect the oral treatment (gavage) with *Stevia rebaudiana* (*Bert.*) *Bertoni* (SRB) and stevioside (STV) on glycaemia and gluconeogenesis of 15 h fasted rats. For this purpose, the rats received SRB (20 mg/kg x day), STV (5.5 mg/kg x day) or an equal volume of water (controls) during 15 days. To measure gluconeogenesis, liver perfusion and isolated hepatocytes were used. Glycaemia and gluconeogenesis from L-alanine (5 mM), L-glutamine (5 mM) and L-lactate (2 mM) were decreased ($P < 0.05$) after pre-treatment with SRB. However, the treatment with STV did not influence glycaemia and gluconeogenesis. Moreover, to get further information about the mechanism by which SRB leaves inhibit gluconeogenesis their potential role as a PPAR γ agonist was investigated. The data showed absence of activation of PPAR γ receptors. In summary, our results showed that the reduction of glycaemia promoted by the treatment with SRB leaves was mediated, at least in part, by an inhibition of hepatic gluconeogenesis. However, this effect did not involve stevioside and the activation of PPAR γ receptors.

Key words: *Stevia rebaudiana* (*Bert.*) *Bertoni*, stevioside, gluconeogenesis, Glycaemia, PPAR γ agonist.

HYPOLYCEMIC EFFECT OF THE HYDROALCOHOLIC EXTRACT OF LEAVES OF *AVERRHOA CARAMBOLA L.* (*OXALIDACEAE*).

ABSTRACT

The effect of the oral treatment (20 mg/kg x day) with hydroalcoholic extract of leaves of *Averrhoa carambola L.* (HELAC) on fasting glycaemia (15 hours) was examined. For this purpose, rats that received vehicle (Control group) or HELAC (HELAC group) during 15 days were compared. HELAC group showed lower fasting glycaemia ($p<0.05$). In contrast, the liver glucose production from L-alanine (5 mM) was increased ($p<0.05$). This effect was mediated, at least in part, by an activation of the catabolism of L-alanine inferred by the increased hepatic urea ($p<0.05$) and L-lactate ($p<0.05$) production. Differently of L-alanine, the glucose production from L-glutamine (5 mM), L-lactate (2 mM) and glycerol (2 mM) was similar to control (Control group vs. HELAC group). In addition, the effect of the HELAC treatment on glucose uptake, inferred by glycogen synthesis and [¹⁴C]-lactate production in soleus muscles were investigated and showed similar results in both groups. Thus, we can conclude that the reduction of glycaemia promoted by the treatment with HELAC was not mediated by an inhibition of hepatic gluconeogenesis and/or an increased glucose uptake by muscles.

Key words: *Averrhoa carambola L.* (*Oxalidaceae*), Carambola, Hepatic Gluconeogenesis, Antidiabetic plants, Hypoglycemic effect.

EFEITOS COMPARATIVOS DE FOLHAS DE STEVIA REBAUDIANA (BERT.)

BERTONI E ESTEVIOSÍDEO NA GLICEMIA E GLICONEOGÊNESE HEPATICA.

RESUMO

O objetivo do presente estudo foi comparar o efeito do tratamento via oral com *Stevia rebaudiana* (*Bert.*) *Bertoni* (SRB) e esteviosídeo (STV) sobre a glicemia e gliconeogênese de ratos em jejum de 15 h. Para alcançar este propósito, os ratos receberam SRB (20 mg/kg x dia), STV (5.5 mg/kg x dia) ou volume equivalente de água (controle) durante 15 dias. Para avaliar a gliconeogênese, perfusão de fígado e hepatócitos isolados foram utilizados. Observou-se redução da glicemia e da gliconeogênese a partir de L-alanina (5 mM), L-glutamina (5 mM) e L-lactato (2 mM) ($P < 0,05$) após tratamento com SRB. Contudo, o tratamento com STV não influenciou a glicemia e a gliconeogênese. Além disso, visando alcançar maiores informações sobre o mecanismo pelo qual folhas de SRB inibem a gliconeogênese, a possibilidade de ação como agonista de receptores PPAR γ foi investigada. Os resultados demonstraram ausência de ativação de receptores PPAR γ . Em resumo, nossos resultados mostraram que a redução da glicemia promovida pelo tratamento com folhas de SRB era mediada, pelo menos em parte, por uma inibição da gliconeogênese hepática. Todavia, este efeito não envolveu a participação do esteviosídeo ou a ativação de receptores PPAR γ .

Palavras-chave: *Stevia rebaudiana* (*Bert.*) *Bertoni*, Esteviosídeo,

Gliconeogêneses, Glicemia, Agonista de PPAR γ .

EFEITO HIPOGLICÊMICO DO EXTRATO HIDROALCOÓLICO DE FOLHAS DE *AVERRHOA CARAMBOLA L.* (*OXALIDACEAE*).

RESUMO

O efeito do tratamento via oral (20 mg/kg x day) com extrato hidroalcoólico de folhas de *Averrhoa carambola L.* (EHFAC) sobre a glicemia de jejum (15 h) foi examinada ao compararmos ratos que receberam veículo (Grupo controle) ou EHFAC (Grupo EHFAC) durante 15 dias. O grupo EHFAC apresentou menor glicemia de jejum ($p<0,05$). Em contraste, a produção de glicose hepática a partir de L-alanina (5 mM) no grupo EHFAC estava aumentada ($p<0,05$). Este efeito foi mediado, pelo menos parcialmente, pela ativação do catabolismo da L-alanina, inferido pela maior produção hepática de uréia ($p<0,05$) e L-lactato ($p<0,05$). Diferentemente da L-alanina, a produção hepática de glicose a partir de L-glutamina (5 mM), L-lactato (2 mM) e glicerol (2 mM) nos grupos controle e EHFAC foi similar. Adicionalmente, o efeito do tratamento com EHFAC sobre a captação de glicose, inferida pela síntese de glicogênio e produção de [14C]-lactato no músculo soleus foi investigado, verificando-se resultados semelhantes nos dois grupos. Assim, concluiu-se que a redução da glicemia promovida pelo tratamento com EHFAC não foi mediada por inibição da gliconeogênese hepática e/ou aumento da captação muscular de glicose.

Palavras-chave: *Averrhoa carambola L.* (*Oxalidaceae*), Carambola, Gliconeogênese Hepática, Plantas antidiabéticas, Efeito hipoglicemiante.

Comparative Effects of *Stevia rebaudiana* (Bert.) Bertoni leaves and Stevioside on Glycaemia and Hepatic Gluconeogenesis.

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Abstract

The purpose of the present study was to compare the effect the oral treatment (gavage) with *Stevia rebaudiana* (Bert.) Bertoni (SRB) and stevioside (STV) on glycaemia and gluconeogenesis of 15-h fasted rats. For this purpose, the rats received SRB (20 mg/kg x day), STV (5.5 mg/kg x day) or an equal volume of water (controls) during 15 days. To measure hepatic gluconeogenesis, liver perfusion and isolated hepatocytes were used. Glycaemia and gluconeogenesis from L-alanine (5 mM), L-glutamine (5 mM) and L-lactate (2 mM) were decreased ($P < 0.05$) after pre-treatment with SRB. However, the treatment with STV did not influence glycaemia and gluconeogenesis. Moreover, to get further information about the mechanism by which SRB leaves inhibit gluconeogenesis their potential role as a PPAR γ agonist was investigated. The data showed absence of activation of PPAR γ receptors. In summary, our results showed that the reduction of glycaemia [promoted by the treatment with SRB leaves was mediated, at least in part, by an inhibition of hepatic gluconeogenesis. However, this effect did not involve stevioside and the activation of PPAR γ receptors.

Key words: *Stevia rebaudiana* (Bert.) Bertoni – stevioside – gluconeogenesis – glycaemia – PPAR γ agonist.

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Introduction

Native of north-eastern Paraguay and southern Brazil, the leaves of *Stevia rebaudiana (Bert) Bertoni* (SRB) have been used by the Guarani Indians to treat diabetes and this popular knowledge has been passed by oral tradition for many centuries.

This empirical knowledge led researchers to study the antidiabetic properties of this plant. The reports about the influence of SRB upon diabetes started with Oviedo et al. [1], who first demonstrated the hypoglycaemic effect of SRB leaves. From this study, several publications, including our previous investigations in rats [2] and human [3] confirmed that the treatment with SRB leaves could reduce fasting glycaemia.

Several reports [4], [5], [6], [7], [8], [9] suggest that the effect of SRB leaves on glycaemia could be mediated, at least in part, by the main sweet component, i.e., stevioside (STV). However, the hypoglycaemic effect of oral STV is still controversial, because STV is degraded by intestinal microflora of rats [10], pigs [11] and human [10] to the diterpenoid aglycone steviol. However, the presence of steviol in the blood after oral ingestion of stevioside was not confirmed [12].

Therefore, considering that the effect of SRB leaves on fasting glycaemia mediated by STV was not well established, the present study was undertaken to investigate this possibility. Moreover, the role of liver gluconeogenesis, that is crucial for the maintenance of fasting glycaemia [13], and the participation of

peroxisome proliferator-activated receptors gamma (PPAR γ), a nuclear mediator of insulin sensitivity [14], [15] were examined. For this purpose, the experiments were executed by using 15-h fasted rats, which represents a favourable system for gluconeogenesis [13] and reporter gene transcription assays modulated by PPAR γ in mammalian cells [16].

Materials and Methods

Plant material: SRB leaves and stevioside

Dried powdered leaves manufactured under the highest quality control were obtained from Steviafarma (Maringá, Brazil). From these dried powdered leaves standardised stevioside was obtained by a method that produces a mixture of stevioside and rebaudioside [17].

Animals and treatment protocol

Male Wistar rats (Maringá, Brazil) weighing about 220 g were employed. The manipulation of the animals followed the Brazilian Law on the protection of animals.

Experimental groups received 20 mg/kg \times day SRB (SRB group) or 5.5 mg/kg \times day STV (STV group) dissolved in water and administered (gavage) at 5:00 p.m. during 15 days. Control groups received water (gavage). During the treatment food and water were freely available.

Determination of glycaemia

After 15 days of treatment food was withdrawn and the rats were killed by decapitation 15-h later. Blood was immediately collected, centrifuged and the glycaemia was determined [18].

Liver perfusion experiments

After 15 days of treatment food was withdrawn and the rats were anaesthetised by an i.p. injection of sodium pentobarbital (40 mg/kg) and submitted to laparotomy. The livers were perfused *in situ* using Krebs Henseleit bicarbonate buffer (KHB), pH 7.4, saturated with O₂/CO₂ (95/5%). The perfusion fluid was pumped through a temperature controlled (37°C) membrane oxygenator prior to entering the liver via portal vein. The perfusion was performed in an open system, without recirculation of the perfusate. A constant flow rate in each experiment was adjusted according to the liver weight (4 mL/g of tissue fresh weight × min).

Fig. 1 illustrates the result of a liver perfusion experiment in which the protocol described above was employed. As shown in Fig. 1 after a pre-perfusion period (10 min), the gluconeogenic substrate was dissolved in the perfusion fluid and infused between the 10th and 30th min of the perfusion period, followed by a post-infusion period (20 min) to allow the return to basal levels.

Samples of the effluent perfusion fluid were collected at 2-min intervals and the glucose concentration was analysed [18]. The differences in the glucose production during (10-30 min) and before (0-10 min) the infusion of glycerol allowed calculation of the areas under the curves (AUC), expressed as µmol/g.

Moreover, similar procedures were performed when L-glutamine (5 mM), L-alanine (5 mM) and L-lactate (2 mM) were used as the gluconeogenic substrates. During the liver perfusion experiments, L-lactate [19], pyruvate [20] and urea [21] production were also measured. Thus, the AUC of Tables 1-4 was obtained from similar experiments as shown in Fig. 1.

Isolation and incubation of isolated hepatocytes

The isolation of hepatocytes was executed by a technique employing collagenase first described by Berry & Friend [22] and modified by Bazotte [23]. For this purpose 15-h fasted rats were anaesthetised and submitted to laparotomy. The liver perfusion started with a Ca⁺⁺ free KHB. Afterwards, this perfusion fluid was replaced by KHB containing collagenase (0.035%) and Ca⁺⁺ (2.5 mM). In this phase the perfusion was done with recirculation of the perfusate that returned to the reservoir and was recirculated. After 5-10 min, the fluid commenced to ooze freely from the surface of the liver. Within 10-30 min, the loss of fluid from the liver surface was too great for the perfusion to be maintained. At this point the consistency of the liver was so soft that it disintegrated on pressure unless handled very gently. The liver was then carefully removed, transferred to a Petri dish with 10-20 mL of enzyme medium at 4°C, and broken up with a blunt spatula. After the filtration and centrifugation, we obtained a supernatant with 95-98% of isolated hepatocytes that, upon microscopic examination (Newbauer chamber), did not stain with trypan blue. The isolated hepatocytes (2×10^6 cells/mL) obtained by the technique described above were incubated during 60 min with KHB (5 mL) containing L-alanine (5 mM), L-Lactate (2 mM), L-glutamine (5 mM), glycerol (2

mM) or no substrate (to get basal values). All flasks were gassed (O_2/CO_2 : 95/5 %) and maintained in a shaking water bath at 37°C during the incubation period. Lastly, the incubation medium was centrifuged and the supernatant was used for glucose [18] determination.

Cell Culture, Transfection and PPAR reporter transactivation assay

Human promonocyte U937 cells (Cell Culture Facility, University of California; San Francisco. CA USA) were maintained on 37°C, CO₂ (5%) subcultured in RPMI-1640 (GIBCO; Grand Island, NY, USA) medium with foetal calf serum (10%), glutamine (2mM), penicillin (50 units/mL) and streptomycin (50 µg/ mL). For transfection assays, the cells were collected by centrifugation and resuspended in transfection solution (0.5 mL/ 1.5 × 10⁷ cells) containing PBS (phosphate-buffered saline), Ca⁺⁺ (100 mM) plus dextrose (0.1%) and mixed with 1.5 µg of human PPAR γ expression vector (provided by J. Magae, Japan) [16] and 3 µg of the luciferase (Luc) reporter (provided by J. Magae, Japan). This reporter plasmid has a synthetic PPAR γ response element, containing one copy of DR-1 (5'- agttcaggtcagaggtcagag -3') cloned immediately upstream of a minimal thymidine kinase (tk) promoter (-32/+45) linked to luciferase coding sequences. Afterwards, the cells were transferred to a cuvette and electroporated using a Bio-Rad gene pulser at 300 mV and 950 µF. Immediately after electroporation, the cells were transferred to fresh RPMI-1640 medium, plated in a 12-well dish and treated in triplicates with ethanol:DMSO 1:1 (vehicle), Pioglitazone (Sigma; St. Louis, MO, USA) at 10⁻⁵ M (PPAR γ agonist) and lyophilized SRB leaves. After 24-h, cells were collected by centrifugation, lysed by the addition of 150 µmL 1X lysis buffer (Promega; Madison, WI, USA) and assayed

for luciferase activity (kit from Promega Corp.). Transfection experiments were performed at least three times. Fold activation was calculated using the ratio of luciferase numbers obtained by the samples treated with ligands with the templates treated with vehicle. Finally, to the transfection assay [24], 1g of dried and pulverised leaves was heated/boiled for 5 min in 100 mL of water, the decoction was, allowed to stand for 30 min and then filtered it through a paper filter. Then, the decoction was lyophilised, and this material was kept at -20°C until use.

Statistical Analysis

The GraphPad Prism program (version 2.0) was used to calculate the areas under the curves (AUC). Data were analysed statistically by the unpaired Student-t test and ANOVA. A 95% level of confidence ($P < 0.05$) was accepted for all comparisons. Results were reported as means \pm SEM.

Results and Discussion

There are few studies showing the effectiveness of herbal therapy for the management of diabetes [25]. In this context, our data shown quite clearly that SRB leaves, but not stevioside, orally-administered during 15 days decreased ($P < 0.05$) glycaemia (Table 1).

In contrast to our results, Jeppesen et al. [6] demonstrated favourable effects of stevioside on glycaemia. Since we used approximately the same daily

dose, such discrepancy could be attributed to several possibilities: 1) the experimental model (Wistar rats vs. type 2 Goto-Kakizaki rats); 2) the period of treatment (2 weeks vs. 6 weeks); 3) the parameter evaluated (fasting glycaemia vs. glucose tolerance test) etc. Another possibility not mentionet by Jeppesen et al. [6] could be the composition of the stevioside.

Since hepatic gluconeogenesis is crucial for glucose maintenance during fasting [13] this metabolic pathway was investigated in livers from rats treated with SRB leaves (SRB group) and the results were compared with livers of rats which received an equal volume of water (control group).

The SRB group showed lower liver glucose production ($P < 0.05$) from L-alanine, whereas the catabolism of L-alanine, inferred from the production of urea, pyruvate and L-lactate was not modified (Table 2). Thus, the possibility of a decreased glucose production from the pyruvate step should be considered. In conformity with this proposition, livers from SRB group exhibited higher ($P < 0.05$) pyruvate and lower ($P < 0.05$) glucose production from L-lactate (Table 3). These results suggest that gluconeogenesis from the pyruvate step was decreased in the livers from the SRB group. Furthermore, lower glucose production ($P < 0.05$) was also obtained when L-glutamine was used as glucose precursor (Table 4).

In contrast to L-alanine, L-lactate and L-glutamine the glucose production from glycerol was not affected by the treatment with SRB leaves (Table 4).

The results obtained from the liver perfusion experiments were summarised in the Fig. 2. Starting with L-alanine and L-lactate that enter in the gluconeogenic pathway at pyruvate step, our results suggest that pyruvate carboxylase (PC) and phosphoenol pyruvate carboxylase (PEPCK) could be inhibited by the treatment

with SRB leaves. According to this view, a recent investigation showed that the PEPCK expression could be influenced by SRB [26]. In agreement, glucose production from L-glutamine, which enters in the gluconeogenic pathway before the PEPCK step, was also inhibited by the treatment with SRB leaves. However, gluconeogenesis was not totally inhibited since the glucose production from glycerol, which enters in this metabolic pathway after the PEPCK step was not influenced by the treatment with SRB leaves.

In contrast to SRB leaves, we did not observe decreased glucose production in livers from animals treated with stevioside (results not shown), even when isolated hepatocytes were employed (Fig. 3).

Because PPAR γ agonists reduce insulin resistance and inhibited the expression of the rate-limiting enzyme of gluconeogenesis, i.e., PEPCK [27], the possibility of an effect on PPAR γ receptors was investigated. Pioglitazone, a synthetic agonist of PPAR γ , that has been used to improve insulin sensibility in patients with type 2 diabetes by improving the cellular signalling triggered by this hormone, was used as a positive control. As shown by Fig. 4, in contrast to the PPAR γ agonist pioglitazone, which increased PPAR γ transcription activity in 4.8 fold, extracts from SRB leaves did not show any effect on PPAR γ . In addition, similar results were obtained when STV was tested (not shown).

Our results confirmed the popular reputation of SRB leaves to reduce glycaemia. But, the possibility of effects on the reproductive system must be considered [28], [29], [30]. On the other hand, these studies were contradicted by

Shiotsu [31] who did experiments using methods as similar as possible to the method used by Mazzei-Planas and Kuc [28].

In view of the fact that SRB leaves contain diterpene glycosides (stevioside, rebaudiosides A, B, C, D, steviobioside etc) and a thousand of other compounds, including non-glycosides diterpernes, sterols, triterpenoids, flavonoids, coumarins, caffeic acid, chlorogenic acid and a variety of volatile oil [32], the identification of the hypoglycemic molecule and/or fraction and the demonstration of the absence of toxicological potential will be necessary to open the possibility for the development of a new antidiabetic compound.

Taken together, our results showed that SRB leaves diminish the glycaemia and one of the mechanisms involved was the reduction of hepatic gluconeogenesis. Furthermore, the hypoglycemic effect promoted by the treatment with SRB leaves was not mediated by PPAR γ activation or stevioside and future studies will test new compounds and/or fractions from SRB leaves.

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Table 1 Effect of the treatment with *Stevia Rebaudiana (Bert.) Bertoni* leaves (SRB) or stevioside (STV) on glycaemia (mg/dL). Experimental groups received 20 mg/kg x day SBR (SRB group) or 5.5 mg/kg x day STV (STV group) dissolved in water and daily administered (gavage) at 5:00 p.m. during 15 days. The control groups received an equal volume of H₂O (gavage). Blood samples were collected from 15-h fasted rats.

Parameter	SRB treatment		STV treatment	
	Control	SRB	Control	STV
Glycaemia	94.10 ± 2.99	67.83 ± 5.70*	86.39 ± 4.64	91.50 ± 6.00

*P< 0.05 vs. Control group.

Table 2 Glucose, urea, pyruvate and L-lactate production from L-alanine (5 mM) in isolated perfused liver from 15-h fasted rats that received *Stevia rebaudiana* (Bert.) Bertoni leaves (SRB group) or water (Control group). SRB (20 mg/kg x day) or water was administered by gavage during 15 days. The values of area under the curves (AUC) were calculated as described in Materials and Methods (Fig. 1). The Data are reported as mean \pm SEM of 4-6 experiments.

Parameters	Values of AUC ($\mu\text{mol/g}$)	
	Control	SRB
Glucose	6.08 ± 0.73	$3.45 \pm 0.39^*$
Urea	25.94 ± 1.69	27.55 ± 1.90
Pyruvate	3.51 ± 0.52	4.25 ± 0.61
L-Lactate	5.61 ± 0.88	5.99 ± 1.10

* P< 0.05 vs. Control group.

Table 3 Glucose and pyruvate production from L-lactate (2 mM) in isolated perfused livers from 15-h fasted rats that received *Stevia rebaudiana* (*Bert.*) *Bertoni* leaves (SRB group) or water (Control group). SRB (20 mg/kg × day) or water was administered by gavage during 15 days. The values of area under the curves (AUC) were calculated as described in Materials and Methods (Fig. 1). The data are reported as mean ± SEM of 4-6 experiments.

Parameters	Values of AUC (μmol/g)	
	Control	SRB
Glucose	10.81 ± 1.19	6.50 ± 0.95*
Pyruvate	3.30 ± 0.50	6.75 ± 0.98*

* P< 0.05 vs. Control group.

Table 4 Glucose production from L-glutamine (5 mM) and glycerol (2 mM) in isolated perfused livers from 15-h fasted rats that received *Stevia rebaudiana* (Bert.) Bertoni leaves (SRB group) or water (Control group). SRB (20 mg/kg × day) or water was administered by gavage during 15 days. The values of area under the curves (AUC) were calculated as described in Materials and Methods (Fig. 1). The data are reported as mean ± SEM of 4-6 experiments.

Parameters	Values of AUC ($\mu\text{mol/g}$)	
	Control	SRB
Glucose from L-glutamine	24.82 ± 1.0	$16.93 \pm 1.0^*$
Glucose from glycerol	9.51 ± 0.5	10.51 ± 0.8

* P<0.05 vs. Control group.

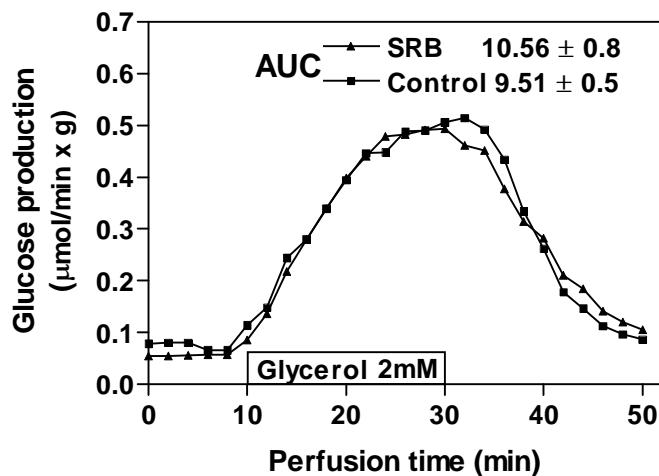


Fig. 1 Demonstrative experiment of glucose production from glycerol in isolated perfused liver from 15-h fasted rats that received during 15 days *Stevia rebaudiana* (Bert.) Bertoni leaves (SRB group) or water (Control group) orally administered. The livers were perfused as described in Materials and Methods. The differences in the glucose production during (10-30 min) and before (0-10 min) the infusion of glycerol allowed calculation of the areas under the curves (AUC), expressed as $\mu\text{mol/g}$.

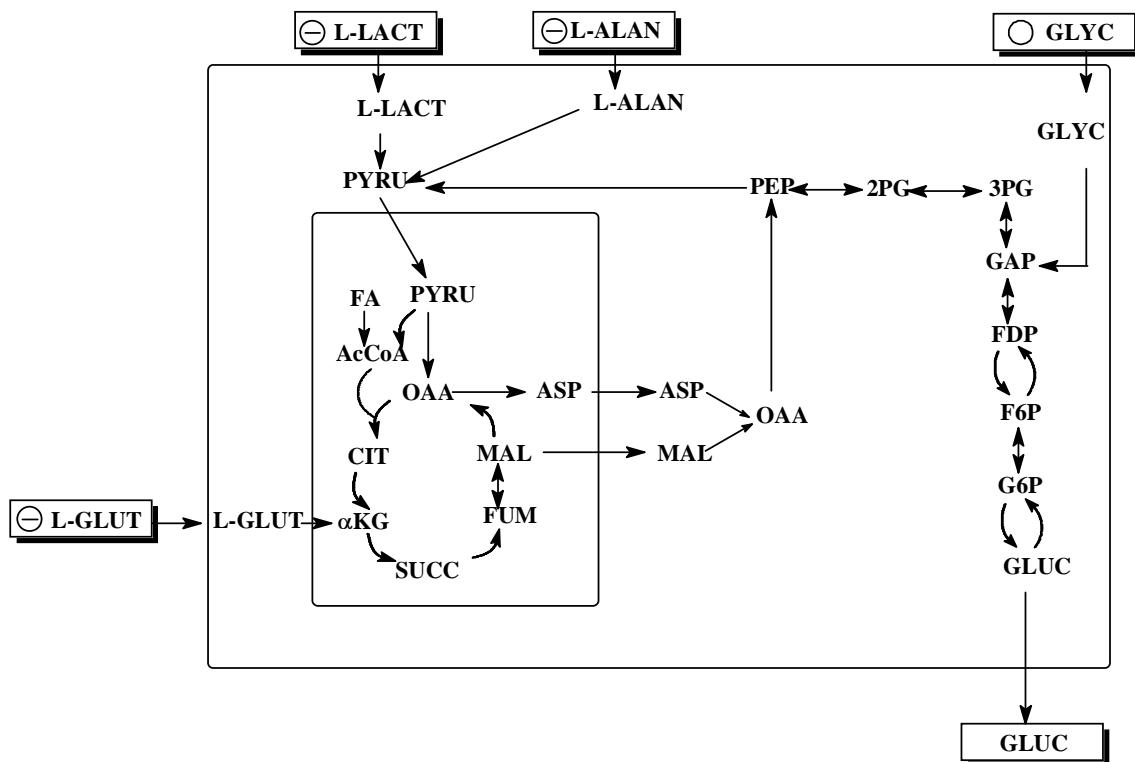


Fig. 2 Effect of the treatment with *Stevia rebaudiana* (Bert.) Bertoni leaves on gluconeogenesis. Plasma membrane is depicted by the large rectangle and the mitochondria by the small rectangle. (⊖) Decreased gluconeogenesis. (○) Absence of effect on gluconeogenesis. Abbreviations: AcCoA, acetyl-CoA; ALAN, L-alanine; ASP, aspartate; CIT, citrate; FA, fatty acid; FDP, fructose diphosphate; F6P, fructose 6-phosphate; FUM, fumarate; GAP, glyceraldehyde phosphate; G6P, glucose 6-phosphate; GLUC, glucose; L-GLUT, L-glutamine; α-KG, α-Ketoglutarate; L-LACT, L-lactate; PYRU, pyruvate; MAL, malate; OAA, oxaloacetate; PEP, phosphoenol pyruvate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; SUCC, succinate.

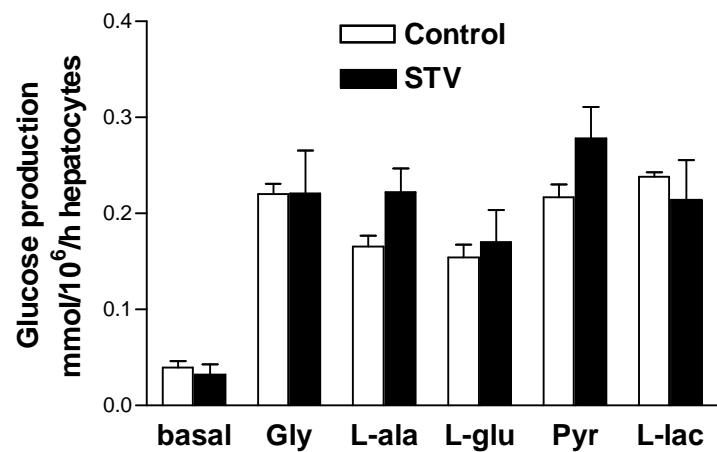


Fig. 3 Glucose production from gluconeogenic substrates in isolated hepatocyte from rats treated with stevioside (STV group) or water (Control group). During 15 days the animals received STV (5.5 mg/Kg x day) (STV group) or an equal volume of water (Control group) by gavage. The data are the mean of 4-5 experiments. Abbreviations: Gly, glycerol (2 mM); L-ala, L-alanine (5 mM); L-glu, L-glutamine (5 mM); Pyr, pyruvate (5 mM) and L-lac, L-lactate (2 mM).

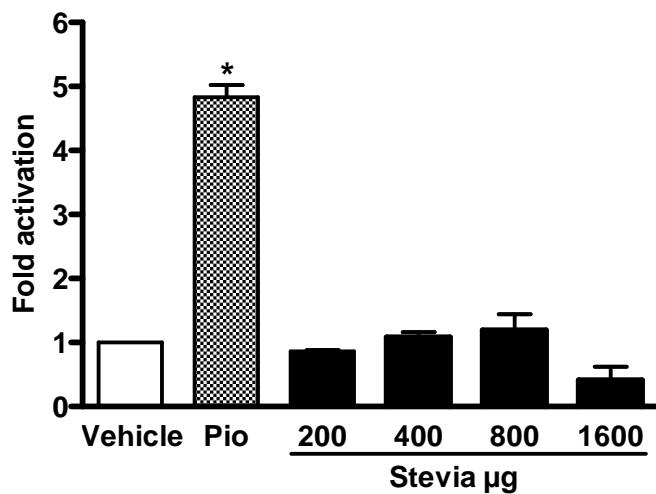


Fig. 4 Effect of pioglitazone (Pio) and increasing amounts of extracts from *Stevia rebaudiana* (Bert.) Bertoni leaves (Stevia) on the activation of PPAR γ .
U937 cells were co-transfected with expression vector encoding the PPAR γ receptor and a reporter vector PPRE-tk-Luc. The transfected cells were treated with vehicle (ethanol:DMSO); Pio at 10^{-5} M; 200, 400, 800 or 1600 μ g of Stevia and assayed for luciferase activity. *p< 0.05 vs Vehicle.

Technical specifications of stevioside		
Definition	Stevioside (stevia extract) is a sweetening agent extracted of the leaves of <i>Stevia rebaudiana</i> (Bert) Bertoni. Stevioside and rebaudioside A are the main components.	
Description	The method for extraction of stevioside was developed in our university (Patent number PI8402752-5-Brazil/ 1984). The stevioside obtained by this method is a slightly yellowish white powder, odorless or having a slightly characteristical odor, 300 times sweeter than sucrose.	
Identification: commercial preparation for human consumption produced by Steviafarma Company (Maringá, PR, Brazil) was employed in this study.		
	Stevioside	Rebaudioside A
Chemical names	19-O-β-glucopyranosyl-13-O[β-glucopyranosyl (1,2)-β-glucopyranosyl]-steviol	19-O-β-glucopyranosyl-13-O[β-glucopyranosyl (1,2)-β-glucopyranosyl (1,3)-β-glucopyranosyl]-steviol
C.A S. Number	57817-89-7	58543-16-1
Chemical formula	C ₃₈ H ₆₀ O ₁₈	C ₄₄ H ₇₀ O ₂₃
Formula Weight	804	960
Structural Formula		
Melting Point	198 °C	242 °C
Specific rotation	[α] _{25,D} -39.3°	[α] _{25,D} -22.0°
Assays:		
Stevioside	72% \pm 2	
Rebaudioside A	23% \pm 2	
Others components of the plant	5 \pm 2	
Moisture	Not more than 3%	
Ash	Not more than 1%	
Density (g/mL)	0.39 a 0.42	
Heavy metals: Arsenic < 1.000 ppm; Leads < 3.600 ppm; Mercury < 0.005 ppm; Chromium < 0.007 ppm; Cadmium < 0.300 ppm		

**Hypoglycemic effect of the hydroalcoholic extract of leaves of
Averrhoa carambola L. (*Oxalidaceae*).**

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ABSTRACT

The effect of the oral treatment (20 mg/kg x day) with hydroalcoholic extract of leaves of *Averrhoa carambola L.* (HELAC) on fasting glycemia (15 h) was examined. For this purpose rats that received vehicle (Control group) or HELAC (HELAC group) during 15 days were compared. HELAC group showed lower fasting glycemia ($p<0.05$). In contrast, livers from HELAC group showed higher ($p<0.05$) glucose production from L-alanine (5 mM). This effect was mediated, at least in part, by an activation of the catabolism of L-alanine inferred by the increased hepatic urea ($p<0.05$) and L-lactate ($p<0.05$) production. Differently of L-alanine, the glucose production from L-glutamine (5 mM), L-lactate (2 mM) and glycerol (2 mM) was similar (Control group vs. HELAC group). In addition, the effect of the HELAC treatment on glucose uptake, inferred by glycogen synthesis and [¹⁴C]-lactate production in soleus muscles were investigated and showed similar results in both groups. Thus, we can conclude that the reduction of glycemia promoted by the treatment with HELAC was not mediated by an inhibition of hepatic gluconeogenesis and/or an increased glucose uptake by muscles.

Key words: *Averrhoa carambola L.* (Oxalidaceae), Carambola, Hepatic Gluconeogenesis, Antidiabetic plants, Hypoglycemic effect.

INTRODUCTION

Averrhoa carambola L. (Oxalidaceae) is known as star fruit and other names specific to different countries. In Brazil this popular tropical fruit is commonly called Carambola, and it is usually consumed as fresh fruit or as juice fruit.

Several studies have reported that patients with chronic renal failure develop severe neurological disturbances after eating carambola fruit or drinking its juice (Neto et al., 1998). This intoxication, caused by a neurotoxin (Carolina et al., 2005) is treated with hemodialysis (Wang et al., 2006). However, in many cases death is not preventable (Chang et al., 2000). In addition, an oxalate nephropathy with permanent renal injury after chronic ingestion of carambola was recently demonstrated (Niticharoenpong et al., 2006). Moreover, the fruit of carambola also promotes an inhibition of human cytochrome P450 3A activity (Hidaka et al., 2004).

On the other hand, studies suggesting toxicity to carambola leaves are absent. In agreement, our previous investigation (Provazi et al., 2001) did not find acute toxicity of carambola leaves in mouse, rats and dogs. In addition, considering that in Brazil carambola leaves are recommended to treat diabetes (Martha et al., 2000) our purpose was investigate the antidiabetic potential of carambola leaves.

Since a hypoglycemic effect was previously demonstrated by using hydroalcoholic extract of leaves of *Averrhoa bilimbi* (Pushparaj et al., 2000), the effect of oral treatment with HELAC on fasting glycemia was examined. Moreover, the effects of HELAC on hepatic glucose production and muscle glucose uptake were investigated.

METHODOLOGY

Plant materials and preparation of the extracts

Averrhoa carambola L. (Oxalidaceae) were collected in Maringá, Paraná State, Brazil (September, 2000), prepared and stored for reference at the State University of Maringá Herbarium (code 8101).

HELAC obtained by a method previously described (Provazi et al. 2005), was lyophilized and dissolved in water immediately before using.

Animals and treatment

Male Wistar rats weighing about 200 g, were individually caged and maintained on standard rodent chow and water *ad libitum*. The manipulation of the animals followed the Brazilian law on the protection of animals.

The rats received HELAC (HELAC group) or vehicle (Control group) at 5:00 pm. during 15 days. The amount (20 mg/kg × day), administration (orally by gavage) and period of treatment were similar to that previously employed (Ferreira et al., 2006) to obtain hypoglycemic effects with *Stevia rebaudiana* leaves.

Determination of glycemia

After 15 days of treatment HELAC and vehicle groups were subdivided in four subgroups: HELAC-Fed, HELAC-Fasted, Control- Fed and Control-Fasted subgroups. Fed subgroups were maintained with free access to food until the

blood collection. Fasted subgroups were maintained without food (15 h) until the blood collection. All rats were killed by decapitation. Blood was immediately collected, centrifuged and the glycemia was determined (Bergmeyer and Bernt, 1974).

Liver perfusion experiments

After 15 days of treatment the rats were anaesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg/Kg) and submitted to laparotomy. The livers were perfused according to the protocol described previously (Ferreira et al., 2006).

All liver perfusion experiments were performed with 15 h fasted rats a favourable condition for gluconeogenesis, at which hepatic glycogen was completely depleted (Batista et al., 1997).

After a pre-infusion period, the gluconeogenic precursor (L-alanine, L-lactate, L-glutamine or glycerol) was dissolved in the perfusion fluid and infused during 20 min, followed by a period of post-infusion to allow the return to basal glucose production. Samples of the effluent perfusion fluid were collected at 2 min intervals and the glucose production was determined (Bergmeyer and Bernt, 1974). The differences in the glucose production during and before the infusion of the gluconeogenic substrate allowed calculating the areas under the curves (AUC), expressed as $\mu\text{mol/g}$.

To obtain the maximal capacity of the liver to produce glucose, liver perfusion experiments using increasing concentrations of L-alanine, L-lactate, L-glutamine and glycerol were performed. The addition of these glucose precursors

increased the rate of glucose production in a dose dependent proportionately to the amount of precursor until the liver capacity was reached, i.e., the lowest concentration at which the maximal hepatic glucose production was obtained. The values obtained to L-alanine, L-lactate, L-glutamine or glycerol were respectively 5 mM, 2 mM, 5 mM and 2 mM (not shown).

Moreover, in part of the liver perfusion experiments L-lactate (Gutmann and Wahlefeld, 1974), pyruvate (Czok and Lamprecht, 1974) and urea (Gutmann and Bergmeyer, 1974) production were measured. Thus, the AUCs shown in the Tables 2-4 were obtained from these liver perfusion experiments.

Soleus muscle experiments

To measure glycogen synthesis and L-lactate production soleus muscles were isolated and incubated as previously described (Massao-Hirabara et al., 2003). Rats were killed by cervical dislocation and soleus muscles were rapidly and carefully isolated, split longitudinally in two equal portions, weighed (20-30 mg) and pre-incubated for 30 min, at 37° C, in Krebs-Ringer bicarbonate buffer pre-gassed for 30 min with O₂/CO₂ mixture (95/5%), containing 5.6 mM glucose, BSA 1%, pH 7.4, with shaking after this period, the muscles were transferred to other vials containing the same buffer, but added with 0.3 µCi/ml D-[U-¹⁴C]-glucose (Amersham 306 mCi/mmol). Incubation was then performed for 1 h under similar conditions, in the presence of vehicle (control), insulin (1 mU/ml) or HELAC. After the incubation period, glycogen synthesis (measured as ¹⁴C

incorporation from [$U-^{14}C$]-glucose) and [^{14}C]-lactate production were measured by liquid scintillation spectroscopy.

Statistical Analysis

Results are reported as means \pm S.D. The program GraphPad Prism was used to calculate the AUC. Data were analyzed statistically by the unpaired Student's t-test or one way ANOVA followed by Tukey test. $p < 0.05$ was accepted for all comparisons.

RESULTS AND DISCUSSION

Since our first study demonstrating antidiabetic properties of *Stevia rebaudiana* (*Bert*) *Bertoni* leaves (Curi et al., 1984), we have been investigating the hypotensive (Ferri et al., 2006), antiobesity (Siqueira et al., 2006), hypolipidemic (Silva et al., 2004) and antidiabetic (Galletto et al., 2004) properties of the Brazilian plants.

The data demonstrated that the treatment with HELAC decreased glycemia ($p < 0.05$) in fasted, but not in fed rats (Table 1). Moreover, the lower glycemia observed with chronic treatment with HELAC, was not observed with one isolated dose of HELAC (not shown).

Because hepatic gluconeogenesis is crucial for glucose maintenance during fasting (Borba-Murad et al., 2005) this metabolic pathway was investigated in livers from rats treated with HELAC (HELAC group) and compared with Control group.

HELAC group showed higher hepatic glucose production ($p<0.05$) from L-alanine (Table 2). This effect was probably the result of the increased catabolism of L-alanine. In agreement the livers from HELAC group showed higher ($p<0.05$) production of urea and L-lactate from L-alanine (Table 2).

Table 1. Effect of the treatment (20 mg/kg × day) with hydroalcoholic extract of leaves of *Averrhoa carambola* L. (HELAC group) or vehicle (Control group) on glycemia (mg/dL). HELAC was administered by gavage at 5:00 p.m. during 15 days. Blood samples were collected from fed and 15 h fasted rats. Data are mean \pm SD of 6-8 animals.

Values of Glycemia (mg/dL)		
	Control Group	HELAC Group
Fed	132.10 \pm 9.03	129.8 \pm 6.54
Fasted	103.9 \pm 5.8	87.1 \pm 4.1 ^a

^a $p<0.05$ as compared with Control group.

The increased availability of L-lactate in the hepatocyte favored gluconeogenesis (Gazola et al., 2007) and helps to explain the largest hepatic glucose production during the infusion of L-alanine. Moreover, this effect was selective to L-alanine, since hepatic glucose production from L-lactate, L-glutamine and glycerol was unchanged (Table 3).

Table 2. Glucose, urea, pyruvate and L-lactate production from L-alanine (5 mM) in isolated perfused liver from 15 h fasted rats that received treatment with hydroalcoholic extract of leaves of *Averrhoa carambola L.* (HELAC group) or vehicle (Control group). Carambola (20 mg/kg × day) or water was administered by gavage during 15 days. The values of the area under the curves (AUC) were calculated as described in Methodology. The data are mean ± SD of 3-6 experiments.

Parameters	Values of AUC ($\mu\text{mol/g}$)	
	Control Group	HELAC Group
Glucose	5.44 ± 0.5	7.69 ± 0.5^a
Urea	22.5 ± 1.3	28.44 ± 2.6^a
Pyruvate	4.96 ± 0.4	4.54 ± 0.1
L-Lactate	4.18 ± 0.1	6.95 ± 0.2^a

^a p<0.05 as compared with Control group.

At this moment its is important to emphasized that the results showed here contrast with the effect of the treatment with *Stevia rebaudiana* where a decreased fasting glycemia was associated with a decreased hepatic gluconeogenesis (Ferreira et al., 2006).

Thus a question can be raised: how can we explain the lower fasting glycemia (Table 1) in rats treated with HELAC if the hepatic glucose production from the main gluconeogenic substrate, i.e., L-alanine (Gazola et al., 2007) was increased. One explanation for this discrepancy could be the fact that the increased glucose production from L-alanine could reflect a compensatory mechanism to protect against hypoglycemia. In agreement with this suggestion a number of investigations have demonstrated an increased gluconeogenesis during hypoglycemia (Davis et al., 1995; Gazola et al., 2007).

Because an increased muscle glucose uptake must be involved in the decreased fasting glycemia showed by HELAC group, this possibility was investigated. The, glucose uptake was inferred by the glycogen synthesis and L-lactate production in isolated soleus muscle. The results showed that in contrast with insulin (positive control), HELAC did not promote activation of glycogen synthesis (Figure 1) and L-lactate production (Figure 2).

Table 3. Glucose production from L-lactate (5 mM), L-glutamine (5 mM) or glycerol (2 mM) in isolated perfused livers from 15 h fasted rats that received treatment with hydroalcoholic extract of leaves of *Averrhoa carambola* L. (HELAC group) or vehicle (Control group). Carambola (20 mg/kg x day) or water was administered by gavage during 15 days. The values of area under the curves (AUC) were calculated as described in Methodology. Data are mean \pm SD of 3-4 experiments.

Parameters	Values of AUC ($\mu\text{mol/g}$)	
	Control Group	HELAC Group
Glucose from L-lactate	11.7 \pm 0.3	12.0 \pm 0.5
Glucose from L-glutamine	17.7 \pm 1.2	17.7 \pm 1.6
Glucose from glycerol	7.2 \pm 0.1	8.7 \pm 1.2

p>0.05 for all comparisons (Control group vs. HELAC group).

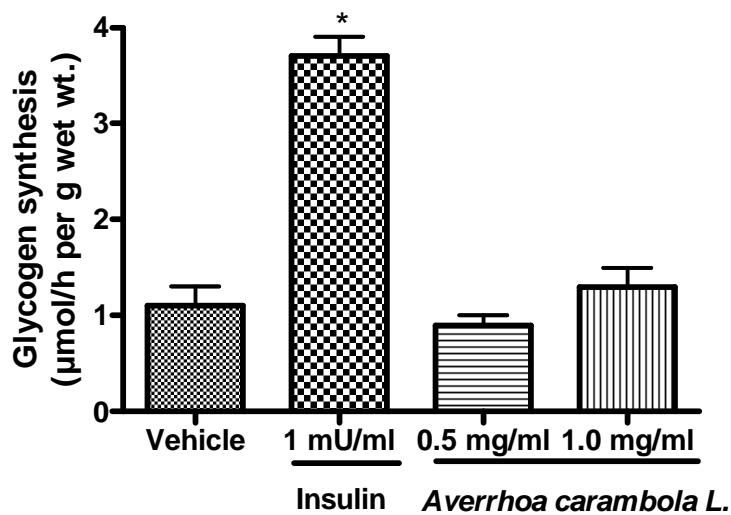


Fig. 1. Effects of vehicle (control group), insulin (1 mU/ml) and hydroalcoholic extract of leaves of *Averrhoa carambola L* (0.5 mg/ml or 1.0 mg/ml) on glycogen synthesis (measured as ^{14}C incorporation from [$\text{U}-^{14}\text{C}$]glucose) in isolated soleus muscle. Values are mean \pm SD ($n = 4$). * $p < 0.05$ compared to control group (one way ANOVA followed by Tukey test).

The results confirmed the popular reputation of carambola to reduce glycemia. Moreover, since HELAC contain triterpenoids, tanines, biogenic amines, amino acids (Provasi et al., 2001) and a thousand of other compounds, the isolation of the hypoglycemic agent will be necessary in future studies.

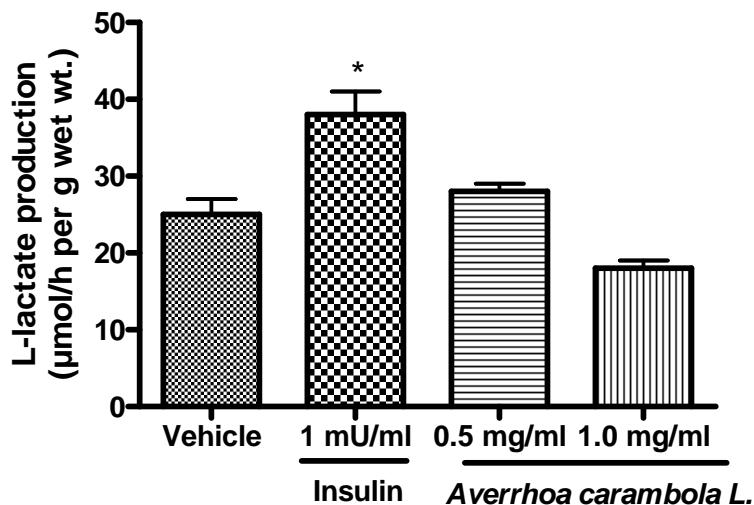


Fig. 2. Effects of vehicle (control group), insulin (1 mU/ml) and hydroalcoholic extract of leaves of *Averrhoa carambola L* (0.5 mg/ml or 1.0 mg/ml) on L-lactate production from glucose 5.6 mM in isolated soleus muscle. Values are mean \pm SD ($n = 4$). * $p < 0.05$ compared to control group (one way ANOVA followed by Tukey test).

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