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Anti-hyperglycemic action of apigenin-6-C-β-fucopyranoside from *Averrhoa carambola*

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ABSTRACT

A stimulatory effect of apigenin-6-C-β-fucopyranoside (1) on glucose uptake was observed when rat soleus muscle was incubated with 1, 10 and 100 μM of this flavonoid glycoside. The presence of specific insulin signaling inhibitors, such as wortmannin, an inhibitor of phosphoinositide 3-kinase (PI3K), RO318220, an inhibitor of protein kinase C (PKC), PD98059, an inhibitor of mitogen-activated protein kinase (MEK), and $HNMPA(AM)_3$, an insulin receptor tyrosine kinase activity inhibitor showed that apigenin-6-C-β-fucopyranoside triggers different metabolic pathways in skeletal muscle. The oral administration of crude extract, fractions and isolated flavonoids (apigenin-6-C-β-fucopyranoside (1) and apigenin-6-C-(2″-O-α-rhamnopyranosyl)-β-fucopyranoside (2)) from *Averrhoa carambola* leaves exhibited a potential hypoglycemic activity in hyperglycemic normal rats. Additionally, both flavonoids significantly increased the muscle and liver glycogen content after an acute treatment. The results indicate that *A. carambola* can be regarded as a potent antihyperglycemic agent with insulin secretagogue and insulin mimetic properties.

1. Introduction

Averrhoa carambola L. (Oxalidaceae), known as star fruit, is native from tropical and subtropical regions of Asia and it was successfully introduced to Brazil in 1817. *Averrhoa carambola* (*A. carambola*) has been used as an appetite stimulant, a diuretic, an antiemetic, an antidiarrheal, an antifebrile and for the treatment of eczemas [1]. Recently, the decoction of *A. carambola* leaves has been used in the treatment of diabetes [2,3]. Other species of the *Averrhoa* genus, such as *Averrhoa bilimbi* Linn. are known for their anti-inflammatory, anti-scorbutic, astringent, anti-bacterial and antidiabetic properties [4]. In addition, a leaf extract and semi-purified fractions of *A. bilimbi* exhibited hypoglycemic and hypolipidemic effects when administered intraperitoneally [5] as well as orally in diabetic rats [6–8].

Some plants used in popular medicine to treat diabetes have been found to contain great amounts of flavonoids showing potentially important antihyperglycemic action, in different *in vivo* and *in vitro* assays, including reduction of serum glucose levels, stimulation of insulin secretion, regulation of enzyme activity in carbohydrate metabolism, stimulation of glucose uptake and glycogen storage in peripheral tissues [9,10]. We have previously demonstrated that flavonoids isolated from

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A. carambola leaves, act on glucose homeostasis through stimulating in vitro glucose uptake and glycogen synthesis. Also, they were able to increase insulin secretion after in vivo treatments [11,12].

Based on the potential antihyperglycemic effect reported for flavonoids, this study was carried out to investigate the effect and the mechanism of action of apigenin-6-C-β-fucopyranoside on ^{14}C-glucose uptake in soleus muscle in normal rats. Also, the acute effect of the crude extract, fractions and isolated compounds from *A. carambola* on serum glucose levels, muscle and liver glycogen content.

2. Materials and methods

2.1. Materials

Tolbutamide and oyster glycogen type II were purchased from Sigma Chemical Company® (St. Louis, MO, USA). The iodine reagents ($CaCl_2 + I_2 + KI$) were purchased from VETEC, Rio de Janeiro, Brazil. Regular human insulin (Biohulin) was obtained from Biobrás, Bioquímica do Brasil S/A (Águas Claras, MG, Brazil). [U-^{14}C]-2-Deoxy-D-glucose (^{14}C-DG), specific activity 10.6 GBq/mmol, D-[^{14}C (U)]-glucose (^{14}C-glucose), specific activity 9.25 GBq/mmol and biodegradable scintillation liquid were obtained from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA). Wortmannin [inhibitor of phosphoinositide 3-kinase (PI3K)], RO318220 [inhibitor of protein kinase C (PKC)], and PD98059 [inhibitor of mitogen-activated protein kinase (MEK)] were purchased from Sigma Chemical Company® (St. Louis, MO, USA). $HNMPA(AM)_3$ [inhibitor of insulin receptor tyrosine kinase activity] was purchased from Enzo Life Sciences® (NY, USA). Salts and solvents were purchased from Merck AG (Darmstadt, Germany).

2.2. Plant material

The leaves of *A. carambola* were collected (March, 2003; co-ordinates −27.687799 latitude; −48.777296 longitude) at Santo Amaro da Imperatriz, Santa Catarina, Brazil and identified by Prof. Daniel de Barcellos Falkenberg. A voucher specimen was deposited at the herbarium of the Botany Department at the Universidade Federal de Santa Catarina, Florianópolis, under number FLOR-24.144.

2.3. Extraction and isolation

The powdered, dried leaves (281 g) were extracted with ethanol–water (EtOH-H_2O) (4:1). The extract was concentrated to dryness by rotatory vaporization at 60 °C under reduced pressure (41.3 g; crude extract-dry leaves ratio = 14.7%). The concentrated extract was then suspended in EtOH-H_2O (4:1) and successively extracted with *n*-hexane, ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH). The ethyl acetate soluble fraction (EtOAc) of 6.9 g was subjected to silica gel (100–200 mesh) CC and eluted with an ethyl acetate/ethanol mixture gradient to afford 36 fractions. Fractions 6–8 (150 mg) and 10–12 (200 mg) were purified by recrystallization from methanol (MeOH) to give pure (HPTLC in ethyl acetate/methanol/acetic acid 80:14:6 and NMR) compounds **1** and **2**, respectively. These compounds were identified by nuclear magnetic resonance spectroscopy (NMR) analysis (^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC) and comparison with literature data [13].

Apigenin-6-C-β-fucopyranoside (compound **1**): Yellow amorphous powder, (drug-extract ratio = 0.36%). ^{1}H NMR (400 MHz, CD_3OD): aglycone moiety δ: 6.61 (s, H-3), 6.53 (s, H-8), 7.84 (d, *J* = 8,0 *Hz*, H-2′ & H-6′), 6.92 (d, *J* = 8,0 *Hz*, H-3′ & H-6′); sugar moiety: 4.64 (d, *J* = 8.4 *Hz*, H-1″), 4.03 (t, *J* = 8.7 *Hz*, H-2″), 3.97 (dd, *J* = 3.0 and 6.5 *Hz*, H-3″), 3.50 (d, J = 3.0 *Hz*, H-4″), 3.17 (m, H-5″), 1.44 (d, *J* = 6.2 *Hz*, H-6″).^{13}C NMR (400 MHz, CD_3OD) aglycone moiety δ: 165.1 (C-2), 99.6 (C-3), 183.1 (C-4), 161.7 (C-5), 108.0 (C-6), 163.9 (C-7), 94.0 (C-8), 161.8 (C-9), 102.8 (C-10), 121.9 (C-1′), 128.3 (C-2′ and 6′), 115.9 (C-3′ and 5′), 157.8 (C-4′); sugar moiety: 71.9 (C-1″), 69.1 (C-2″), 78.5 (C-3″), 70.6 (C-4″), 70.8 (C-5″), 18.1 (C-6″).

Apigenin-6-C-(2″-O-α-rhamnopyranosyl)-β-fucopyranoside (compound **2**): Yellow amorphous powder (drug-extract ratio = 0.48%). ^{1}H NMR (400 MHz, CD_3OD): aglycone moiety δ: 6.61 (s, H-3), 6.54 (s, H-8), 7.85 (d, *J* = 7.6 *Hz*, H-2′ and H-6′), and 6.94 (d, *J* = 7,6 *Hz*, H-3′ and H-6′); sugar moieties: 4.91 (d, J = 9.6 *Hz*, H-1″), 4.27 (t, *J* = 8.8 *Hz*, H-2″), 3.75 (m, H-3″), 3.69 (sl, H-4″), 3.84 (m, H-5″), 1.28, (d, *J* = 6.0 *Hz*, H-6″), 5.18 (sl, H1‴), 3.78 (sl, H-2‴), 3.28 (overlapped with signals of CD_3OD, H-3‴), 3.09 (t, *J* = 9.5 *Hz*, H-4‴), 2.54 (t, *J* = 8.4 *Hz*, H-5‴), and 0.71 (d, *J* = 6.0 *Hz*, H-6‴). ^{13}C NMR (400 MHz, CD_3OD) aglycone moiety δ: 165.1 (C-2), 104.3 (C-3), 183.0 (C-4), 161.6 (C-5), 108.8 (C-6), 163.0 (C-7), 95.0 (C-8), 159.6 (C-9), 102.8 (C-10), 122.0 (C-1′), 128.3 (C-2′ and 6′), 115.9 (C-3′ and 5′), and 157.7 (C-4′); sugar moieties: 72.2 (C-1″), 75.1 (C-2″), 76.5 (C-3″), 72.8

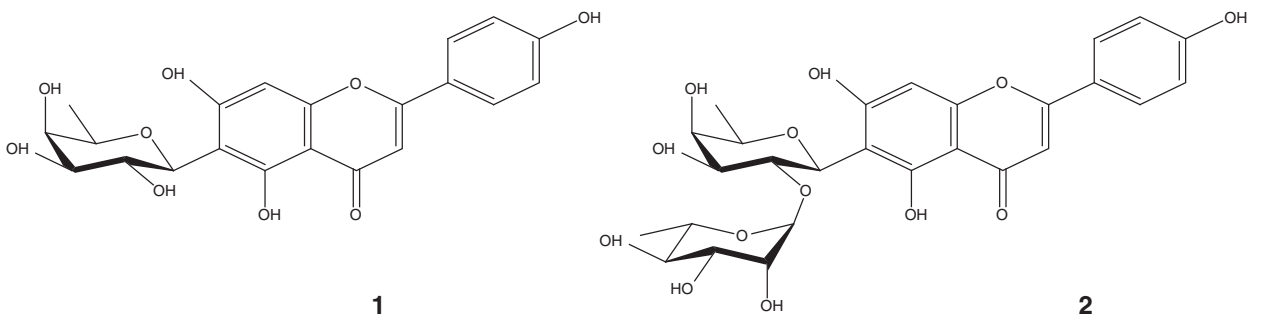

Fig. 1. Chemical structure of compounds apigenin-6-C-β-fucopyranoside (compound **1**) and apigenin-6-C-(2″-O-α-rhamnopyranosyl)-β-fucopyranoside (compound **2**) isolated from the EtOAc fraction of *A. carambola* leaves.

Table 1
Acute effect of lispro insulin, tolbutamide and crude extract of *A. carambola* on the serum glucose level in the oral glucose tolerance curve.

Time	Group I	Group II	Group III	Group IV		
	Vehicle	Hyperglycemic glucose	Hyperglycemic plus tolbutamide	Glucose fed hyperglycemic plus *A. carambola* crude extract		
(min)	(1% EtOH-H_2O)	(4 g/kg)	(100 mg/kg)	200 mg/kg	400 mg/kg	800 mg/kg
0	111.8 ± 2.8	122.6 ± 5.8	108.4 ± 5.1	110.1 ± 3.3	111.8 ± 5.7	125.4 ± 7.8
15	114.7 ± 6.8	196.2 ± 13.6***	168.6 ± 5.6***	178.4 ± 8.7***	174.7 ± 7.3***	172.0 ± 2.8***
30	125.5 ± 3.8	201.7 ± 11.9***	172.0 ± 10.4***#	184.4 ± 7.6***	167.8 ± 7.3***#	182.2 ± 5.8***
60	128.3 ± 3.8	178.4 ± 9.1***	145.8 ± 6.8#	147.5 ± 5.6**#	153.2 ± 5.9***#	165.9 ± 8.9**
120	120.1 ± 6.9	137.8 ± 7.7	131.8 ± 9.5	138.4 ± 6.0	139.8 ± 5.2	151.8 ± 6.7
180	120.7 ± 2.0	134.3 ± 4.4	131.8 ± 6.9	144.1 ± 4.2*	149.5 ± 4.1**	140.4 ± 6.0

Values expressed as mean ± S.E.M.; n = 6 in duplicate for each treatment. Statistically significant difference to the corresponding zero time value; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. Significantly different to the corresponding hyperglycemic group; # $p \leq 0.05$.

(C-4″), 74.6 (C-5″), 16.0 (C-6″), 101.2 (C-1‴) 71.1 (C-2‴), 70.9 (C-3‴), 72.4 (C-4‴), 68.7 (C-5‴), and 16.8 (C-6‴) (Fig. 1).

2.4. Experimental animals

Male Wistar rats (190–220 g) were used. They were bred in our animal facility and housed in an air-conditioned room (approximately 22 ± 2 °C and during the entire experiments) with controlled lighting on a 12:12 h light/dark cycle (lights on from 06:00 to 18:00 h). The animals were maintained with pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil), while tap water was available ad libitum. For all oral treatments, 0.5 mL of each respective substance was given by gavage. Animals described as fasted had been deprived of food for 16 h but allowed free access to water [14]. All the animals were monitored carefully and maintained in accordance with the ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation. (Protocol CEUA/PP007).

2.5. Determination of the serum glucose level

Blood samples (100 μL) were collected from the tail vein of the anesthetized rat, centrifuged and the serum was used to determine the glycemia (GBC 916 UV–visible spectrophotometer) by the glucose oxidase method [15]. The commercial kit used to determine the glycemia was from Gold Analisa (Belo Horizonte, MG, Brazil).

2.6. Study of the effects of crude extract, fractions or isolated compounds from A. carambola on the serum glucose level in the oral glucose tolerance curve

Fasted rats were divided into groups of six animals for each treatment: Group I, normal rats that received vehicle 1% EtOH-H_2O; Group II, hyperglycemic rats that received glucose (4 g/kg) plus vehicle; Group III, hyperglycemic rats that received glucose (4 g/kg) plus tolbutamide (100 mg/kg b.w.) by oral gavage; Group IV, rats that received glucose (4 g/kg) plus crude extract (200, 400 and 800 mg/kg b.w.) (Table 1). As shown in Table 2, the hyperglycemic rats received EtOAc or *n*-BuOH (400 and 800 mg/kg b.w.) fractions. Also, hyperglycemic rats received isolated compounds **1** or **2** (20 and 50 mg/kg b.w.) by oral gavage (Fig. 2). Blood samples were collected just prior to and at 15, 30, 60, 120 and 180 min after the glucose loading and the serum glucose levels were measured.

2.7. Studies on glycogen content

Soleus muscles and livers were harvested from normal fed or fasted rats and hyperglycemic rats treated with compounds **1** or **2** (50 mg/kg) or regular insulin (0.5 IU) and used for the assay of glycogen content immediately after 3 h of

Table 2
Acute effect of EtOAc and *n*-BuOH fractions of *A. carambola* on the serum glucose level in the oral glucose tolerance curve.

Time	Group I	Group II	Group III		Group III	
	Vehicle	Hyperglycemic glucose	Glucose fed hyperglycemic plus *A. carambola* EtOAc fraction		Glucose fed hyperglycemic plus *A. carambola* *n*-BuOH fraction	
(min)	(1% EtOH-H_2O)	(4 g/kg)	400 mg/kg	800 mg/kg	400 mg/kg	800 mg/kg
0	111.8 ± 2.8	122.6 ± 5.8	108.3 ± 3.1	108.2 ± 3.9	134.2 ± 5.4	126.6 ± 3.6
15	114.7 ± 6.8	196.2 ± 13.6***	135.6 ± 5.0##	152.4 ± 9.3#	178.4 ± 5.7	169.2 ± 7.1
30	125.5 ± 3.8	201.7 ± 11.9***	163.1 ± 5.3##	167.8 ± 9.2#	178.7 ± 5.4	162.2 ± 6.6#
60	128.3 ± 3.8	178.4 ± 9.1***	131.3 ± 6.6##	166.5 ± 5.8	150.0 ± 4.9#	148.5 ± 6.2#
120	120.1 ± 6.9	137.8 ± 7.7	113.0 ± 4.8##	142.7 ± 4.9	173.0 ± 5.9	129.5 ± 4.7
180	120.7 ± 2.0	134.3 ± 4.4	102.8 ± 6.1##	130.0 ± 4.3	172.0 ± 4.4	126.2 ± 2.6

Values expressed as mean ± S.E.M.; n = 6 in duplicate for each treatment. Statistically significant difference to the corresponding zero time value; ***$p \leq 0.001$. Significantly different to the corresponding hyperglycemic group; # $p \leq 0.05$; ## $p \leq 0.01$.

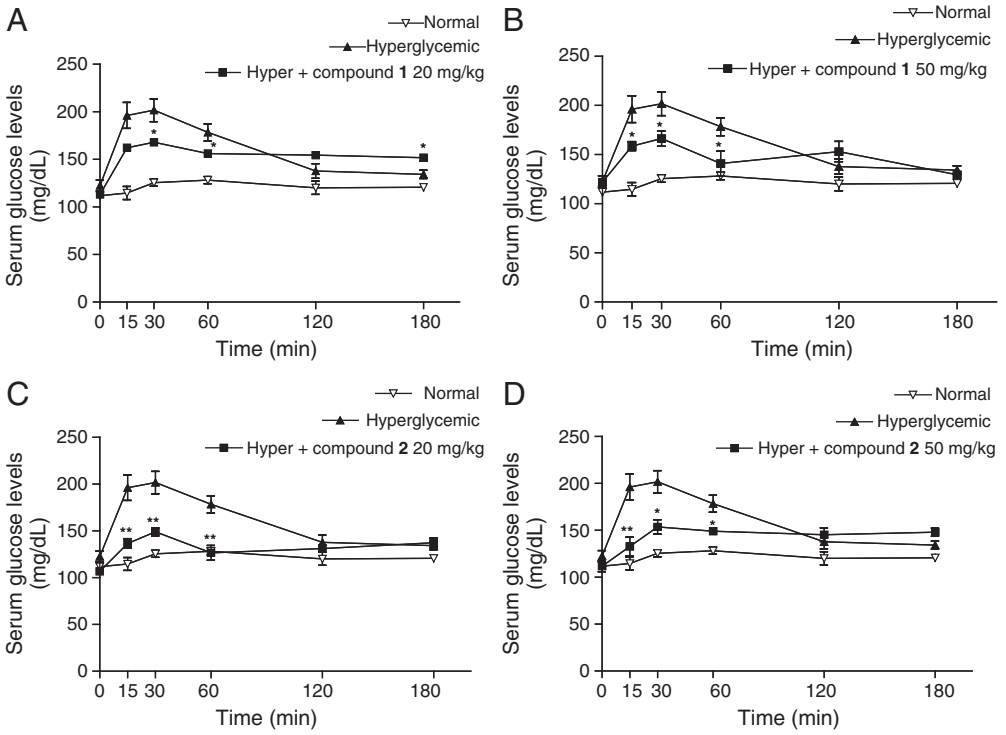

Fig. 2. Effect of compound **1** and compound **2** isolated from *A. carambola* on the oral glucose tolerance curve. Values are expressed as mean ± S.E.M; n = 6 in duplicate for each treatment. Statistically significant difference to the corresponding hyperglycemic group; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

treatment (Fig. 3A and B). Glycogen was isolated from these tissues as described by Krisman, 1962 [16] with minor modifications. The tissues were weighed, homogenized in 33% KOH and boiled at 100 °C for 20 min, with occasional stirring. After cooling, 96% ethanol was added to the samples and heated to boiling followed by cooling in an ice bath to aid the precipitation of glycogen. The homogenates were centrifuged at 1300 × *g* for 15 min, the supernatant was discarded and the pellets were neutralized with saturated NH_4Cl before being heated to 100 °C for 5 min, washed and resolubilized in water. Glycogen content was determined by treatment with iodine reagent and the absorbance was measured at 460 nm. The results were expressed as mg of glycogen/g of tissue.

2.8. Studies on ^{14}C-glucose uptake in rat soleus muscle

For the [U-^{14}C]-2-deoxy-D-glucose uptake experiments, soleus muscles from normal rats were used. Slices of soleus muscle were distributed (alternately left and right) between basal and treated groups. The muscles were dissected, weighed, and preincubated and incubated at 37 °C in Krebs Ringer-bicarbonate (KRb) buffer with a composition of 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, and 25 mM $NaHCO_3$ and bubbled with O_2/CO_2 (95%:5%, v/v) until pH 7.4. Apigenin-6-C-β-fucopyranoside (compound **1**) (1, 10, 100 μM) and insulin (10 nM) were added to the preincubation (30 min) and incubation media (60 min) in the presence or absence of 100 nM wortmannin, 40 μM RO318220 or 50 μM PD98059. ^{14}C-DG (0.1 μCi/mL) was added to each sample during the incubation period. After incubation, the muscle samples were homogenized in 0.5 N NaOH and boiled for 10 min; 25 μL aliquots of tissue and external medium were placed in scintillation liquid on an LKB RackBeta liquid scintillation spectrometer (model 1215; EG and G-Wallac, Turku, Finland), for the radioactivity measurements. The results were expressed as the tissue/medium (T/M) ratio: cpm/mL tissue fluid per cpm/mL incubation medium [14].

2.9. Data and statistical analysis

Data were expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Bonferroni post-test or unpaired Student's *t*-test was used to identify significantly different groups. Differences were considered to be significant at $p \leq 0.05$. The software InStat version 3.05; Graph-Pad Software Inc., San Diego, CA was used for statistical analysis.

3. Results

3.1. Effect of tolbutamide, crude extract, EtOAc, n-BuOH fractions or apigenin-6-C-β-fucopyranoside and apigenin-6-C-(2″-O-α-rhamnopyranosyl)-β-fucopyranoside from A. carambola on the oral glucose tolerance curve

As expected, after starting the glucose tolerance test in normal rats overloaded with glucose, the serum glucose concentration was significantly increased when compared with the zero time of this group. The oral hypoglycemic agent, tolbutamide (100 mg/kg), produced a typical serum glucose lowering at 30 and 60 min compared to the hyperglycemic group. The normal vehicle control group

(1% EtOH-H_2O) showed an unchanged profile of glycemia over the time studied (Table 1).

Crude extract at 200 and 400 mg/kg was effective in reducing glycemia at 30 min and/or 60 min after oral treatment when compared with the respective hyperglycemic control group. Furthermore, oral treatment with 400 mg/kg of the EtOAc fraction in hyperglycemic rats produced a significant antihyperglycemic effect from 15 to 180 min (Table 2). In addition, the glycemia was maintained at basal levels from 60 to 180 min (Table 2). On the other hand, the 800 mg/kg dose showed a fast antihyperglycemic effect only in the first two periods studied (15 and 30 min) (Table 2). Table 2 shows the effect of the *n*-BuOH fraction of *A. carambola* at 400 and 800 mg/kg in hyperglycemic animals. Although both doses showed a slightly antihyperglycemic effect (at 60 or 30 min and 60 min, respectively) neither 400 nor 800 mg/kg doses were as powerful as the EtOAc fraction (400 mg/kg), previously demonstrated in Table 2.

The oral administration of compound **1** reduced serum glucose levels in hyperglycemic rats and the maximum reduction observed was 19% at 15 min with the higher dose (Fig. 2B). The effect of compound **2** on the glucose tolerance curve was similar and is shown in Fig. 2C and D. Oral administration with 20 and 50 mg/kg of this compound significantly reduced the glycemia by around 32%, 24% and 16% at 15, 30 and 60 min, respectively, after treatment with the higher dose used. At 120 and 180 min, glycemic levels were similar to respective results for the hyperglycemic control groups.

3.2. Effect of apigenin-6-C-β-fucopyranoside and apigenin-6-C-(2″-O-α-rhamnopyranosyl)-β-fucopyranoside from A. carambola on the glycogen content of soleus muscle and liver

Fig. 3A and B shows that the glycogen content was significantly increased, 3 h after the administration of glucose (4 g/kg) by oral gavage, in soleus muscle compared with fed and fasted normal rats and in liver when compared with fasted normal rats. In addition, the well known insulin stimulatory effect on glycogen storage in both tissues was observed 3 h after insulin treatment in hyperglycemic normal rats compared with fasted and hyperglycemic normal rats.

Compound **1** was able to significantly increase the glycogen content in soleus muscle when compared with fed normal (4.6 times), fasted normal (10.7 times), hyperglycemic (1.2 times) and hyperglycemic plus insulin (1.14 times) animals 3 h after treatment. Moreover, compound **1** stimulated glycogen content in the liver as well. This change was 25 times when compared

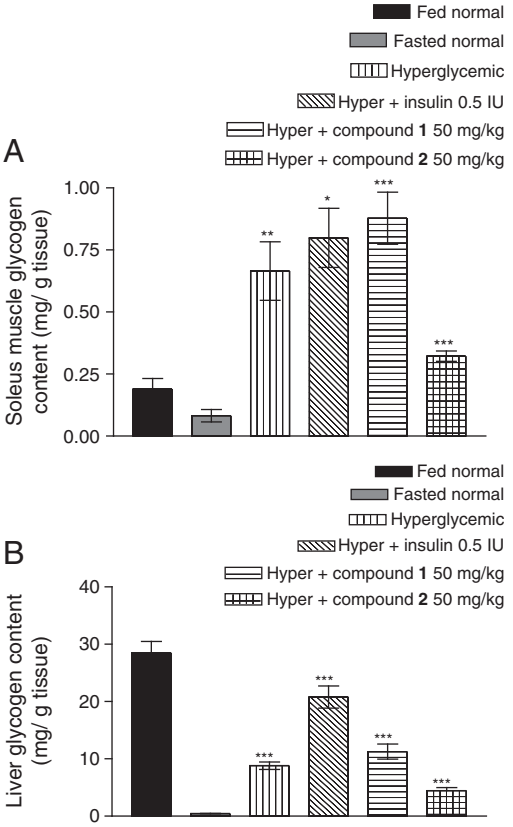

Fig. 3. Effect of compound **1** and compound **2** on the glycogen content in normal hyperglycemic rats. (A) soleus muscle and (B) liver 3 h after treatment by oral gavage. Values are expressed as mean ± S.E.M; n = 6 in duplicate for each group. Significantly different to the corresponding fasted normal group; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

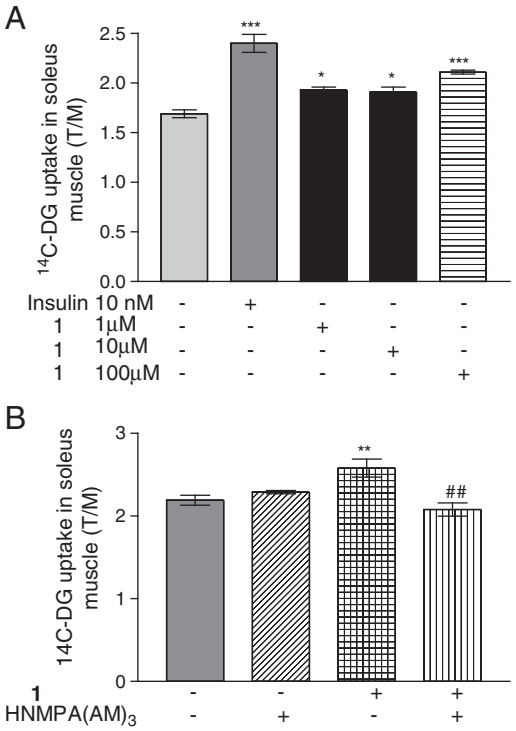

Fig. 4. Concentration–response curve of apigenin-6-C-β-fucopyranoside (compound **1**) (A) and effect of 100 μM $HNMPA(AM)_3$ on the stimulatory action of 100 μM compound **1** (B) on ^{14}C-glucose uptake in rat soleus muscle. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; n = 6 in duplicate for each group. Significant at $^{***}p \leq 0.001$, $^{**}p \leq 0.01$ and $^{*}p \leq 0.05$ in relation to basal group; $^{\#\#}$ $p \leq 0.01$ in relation to compound **1** group.

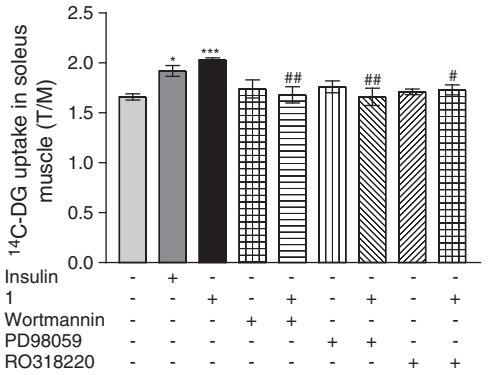

Fig. 5. Effect of enzyme inhibitors, 100 nM Wortmannin, 40 μM RO318220 and 50 μM PD98059 on the stimulatory action of 100 μM compound **1** on ^{14}C-glucose uptake in rat soleus muscle. Basal group = no treatment. Signal (+) and (−) indicate the presence and absence, respectively, of each substance in the incubation medium. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; $n = 6$ in duplicate for each group. Significant to $^{***}p \leq 0.001$, $^{*}p \leq 0.05$ in relation to basal group. Significant to $^{\#\#}p \leq 0.01$ and $^{\#}p \leq 0.05$ in relation to compound **1** group.

with the fasted normal group at 3 h after treatment and it represented 54% of the insulin stimulatory effect.

The stimulatory effect of compound **2** on muscle glycogen content (around 1.7 and 3.9 times) was observed 3 h after treatment when compared with fed and fasted groups, respectively. However, compound 2 did not alter the glycogen content as compared with hyperglycemic group. In the liver, the effect of compound **2** was around 10-fold when compared with fasted normal rats and when compared with insulin, representing 21% of the total stimulatory effect of the hormone.

3.3. Effect of apigenin-6-C-β-fucopyranoside from A. carambola and insulin on ^{14}C-glucose uptake in the rat soleus muscle

Fig. 4A shows the in vitro effect of compound **1** (1, 10 and 100 μM) and insulin (10 nM) on glucose uptake in the rat soleus muscle following 60 min of incubation. As expected, insulin stimulated significantly the ^{14}C-DG uptake when compared to the control group. The stimulatory effect of compound **1** was significant at 1, 10 and 100 μM and represented 14.2, 14 and 25% of glucose uptake compared to the basal value at 60 min, respectively.

Taking into account the effect of compound **1** on glycemia and on glycogen synthesis, we investigated whether the action of compound **1** on glucose uptake also involves the insulin signaling pathways. To do this, we performed the glucose uptake assays in the presence of specific inhibitors of insulin signaling. As observed in Fig. 4B, the stimulatory effect of compound **1** was completely blocked in the presence of 100 μM of HNMPA(AM)$_3$ [17–19]. Additionally, the pretreatment of the muscle with wortmannin (PI3K inhibitor), RO318220 (atypical PKCs inhibitor) and PD98059 (MEK inhibitor) completely blocked the compound **1**-induced glucose uptake (Fig. 5).

4. Discussion

Flavonoids are a large group of phenolic plant constituents and their bioactive potential in the treatment and prevention of diabetes and other diseases has been demonstrated [9,10]. They can affect glucose transport and metabolism in peripheral tissues as well as inducing insulin release from β-cells in the pancreas [14,15,20]. This study showed the antihyperglycemic effect of the crude extract, fractions and isolated compounds of *A. carambola* leaves in normal hyperglycemic rats following an acute treatment. Also it was demonstrated the stimulatory effect of apigenin-6-C-β-fucopyranoside (compound **1**) on glucose uptake in an insulin target, soleus muscle.

Several plants rich in flavonoids have been shown to have an effect on blood glucose levels. Similar results showing the glucose lowering effects of the crude extract of *A. carambola* leaves in hyperglycemic normal rats were reported by Provasi et al. [2]. Also, it has been reported in the literature that the extracts of another *Averrhoa* species, *A. bilimbi*, showed antihyperglycemic effect in normal hyperglycemic and diabetic rats when compared with the respective control groups [6,7]. Furthermore, acute treatment with *Syzygium cordatum* leaf extracts has been found to decrease serum glucose levels in hyperglycemic rats compared to a control group. In addition, the crude extracts from the roots and leaves of *Wilbrandia ebracteata* has shown significant activity in glucose-hyperglycemic rats [21,22]. The EtOAc and *n*-BuOH fractions were isolated from the crude extract and their potential antihyperglycemic effects were studied in normal glucose-fed rats. The antihyperglycemic effect of the EtOAc fraction was more marked than that of *n*-BuOH at 400 mg/kg and was around 26.4% at 60 min after treatment (Table 2). This effect was as pronounced as that caused by tolbutamide (18.26%) (Table 1), a sulfonylurea agent that increases insulin secretion from the pancreas [23]. In line with these results, previous studies of Pushparaj et al. [7] and Tan et al. [8] showed the effect of aqueous and *n*-BuOH fractions from *A. bilimbi* leaves on glucose serum levels in diabetic and hyperglycemic normal rats. Both fractions improved the glucose tolerance curve in diabetic rats and hyperglycemic rats. Furthermore, after two weeks of treatment the fractions increased plasma insulin levels when compared with the zero time and with the diabetic control group.

Considering the EtOAc fraction's antihyperglycemic effect, two glycosylated flavonoids, compound **1** and compound **2** (C-flavones) were isolated from this fraction. Both flavonoids were previously demonstrated to act through at least two different mechanisms, stimulating insulin secretion from the pancreas and interacting with classical cellular insulin metabolic signaling [11,12]. The oral administration of compounds **1** and **2** at 20 and 50 mg/kg resulted in significant antihyperglycemic effects in glucose-fed normal rats. Recently, Hsu et al. [24] demonstrated the antihyperglycemic effect of puerarin, an isoflavone, in normal rats, hyperglycemic normal rats and diabetic rats. Puerarin reduced glycemia in normal and diabetic rats in a dose dependent manner and it was also able to attenuate the increase of plasma glucose induced by an intravenous glucose challenge in normal rats.

The antihyperglycemic effect of fractions from *Cephalotaxus sinensis* leaves were evaluated in streptozotocin (STZ)-induced

diabetic rats. Three main flavonoids were isolated and identified from the most active fraction, apigenin-5-O-[α-L-rhamnopyranosyl-(1→4)-6-O-β-D-acetylglucopyranoside], apigenin and apigenin-5-O-[α-L-rhamnopyranosyl-(1→4)-6-O-β-D-glucopyranoside]. These flavonoids were shown to significantly increase the GLUT-4 protein level in membrane preparations from mice adipocytes which could contribute to the effect of *C. sinensis* on glucose homeostasis [25]. We have demonstrated the antihyperglycemic effect of the EtOH fraction, rich in glycosylated flavonoids, from *Bauhinia forficata* leaves as well as the major flavonoid of that fraction, kaempferitrin [26,27]. Recently, the flavonoid rich fraction of *Pilea microphylla*, constituted mainly by apigenin-7-O-glucoside, quercetin, rutin and luteolin-7-O-glucoside was shown to improve oral glucose tolerance in normal and STZ-diabetic rats [28]. Also, the antihyperglycemic activity of plant extracts rich in flavones in hyperglycemic and diabetic rats has been described [29,30]. The *n*-butanol fraction and the methanol subfraction from *W. ebracteata* and the *C*-glycosylflavones isovitexin and swertisin showed a potent antihyperglycemic action when compared with the crude extract and with the controls and this effect seems to be mediated through insulin secretion from the pancreas [22].

Recently, the flavonoids apigenin and apigenin-7-O-glucoside showed an antihyperglycemic effect and also reduced the glycemia of streptozotocin induced-diabetic rats after 7 days of treatment [31]. Furthermore, it has been demonstrated that apigenin had a protective effect on pancreatic β-cell destruction in a model of Sterptozotocin-induced diabetes and significantly increased insulin release [32]. The results observed in vivo for compounds **1** and **2** may be due to the stimulus of pancreatic function such as the insulin secretion as well as their action as insulin mimetic agents as previously proposed [11,12].

In mammals, carbohydrate is stored mainly in the form of glycogen, with skeletal muscle and liver as the major storage sites. Glycogen metabolism is regulated by insulin/glucagon through activation and/or inhibition of several enzymes and proteins [33]. The determination of glycogen levels in muscle and liver of hyperglycemic normal rats after acute treatments with compounds **1** and **2** revealed a significant increase in glycogen content when compared with fasted normal rats (Fig. 3A and B). Additionally, the known effect of insulin on muscle and hepatic glycogen storage was observed. The study demonstrated that the stimulatory effect of compound **1** on glycogen storage in soleus muscle as well as in liver was greater than that of compound **2** at the same dosage (50 mg/kg). Such an effect of compound **1** was quite similar to that of insulin in muscle. Also, we demonstrated that compound **1** stimulated glucose uptake in soleus muscle and this effect was mediated through the classical insulin signaling since in the presence of specific insulin signaling inhibitors (PI3-K, atypical PKCs and MEK) the stimulatory effect of the flavonoid was completely abolished.

Flavonoids and plant extracts with proven antihyperglycemic activity have been shown to influence glucose uptake, glycogen synthesis and glycogen deposition in different tissues as well as to interact with key enzymes of the glycolytic route in rats [14,34]. We have demonstrated that both flavonoids from *A. carambola* are able to increase insulin secretion and to influence glucose disposal acting through insulin signaling and increasing glucose uptake and glycogen synthesis in soleus muscle. Furthermore, Zanatta et al. [35] showed that kaempferol 3-neohesperidoside, a flavonol isolated from *Cyathea phalerata*, increased muscle glycogen content in diabetic rats and stimulated glucose uptake in normal rats. The results found for compound **2** and especially for compound **1** (Figs. 2 and 3) are closely correlated with the known insulin activity on the stimulatory effect of glucose disposal and lowering serum glucose levels in normal hyperglycemic rats (Fig. 3). As previously shown and in the present study we can propose that the *in vivo* effect of crude extract, fractions and isolated compounds from *A. carambola* is a consequence of direct peripheral glucose uptake, glycogen synthesis and insulin secretion or a combination of both.

5. Conclusions

We have shown that apigenin-6-C-β-fucopyranoside was able to increase glucose uptake in soleus muscle acting through insulin signaling pathways such as insulin receptor tyrosine kinase activity, PI3K, atypical PKCs and MEK. Also, both flavonoids reduced serum glucose levels after acute treatments being able to manage glucose utilization through different pathways. In conclusion, our results indicate that *A. carambola* should be regarded as a potent antihyperglycemic agent with insulin secretagogue and insulin mimetic properties being an attractive adjuvant for the treatment of diabetic patients in the future.

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